

REMARKS

Claims 25-28 are pending in this application. New claims 35-40 are added herein. No new matter is added by these claims and they are supported at pages 58-59, 109, and 141 of the specification and at Figures 31 and 32 and SEQ ID NOS: 82 and 83.

Applicants have cancelled claims 22-24 and 29-34 herein without prejudice or disclaimer.

Claim 25 has been rewritten in independent form. Claims 26 and 27 have been amended so that they depend from claim 25, rather than from cancelled claim 22.

Applicants respectfully request that the Examiner consider the following remarks in response to the Office Action.

Priority Determination:

The Examiner states that Applicants have not complied with one or more conditions for receiving the benefit of an earlier filing date under 35 U.S.C. § 119 (e) or § 120.

Specifically, the Examiner notes that "[a]n application in which the benefits of an earlier application are desired must contain a specific reference to the prior application(s) in the first sentence of the specification of (sic) in an application data sheet (37 CFR 1.78(a)(2) and (a)(5))."

Applicants' Representative thanks the Examiner for the helpful and courteous discussion held on August 2, 2004. During that conversation, Applicants' Representative respectfully directed the Examiner's attention to the United States Patent and Trademark Office Official Gazette Notice of 18 March 2003 (enclosed as Appendix 1 for the convenience of the Examiner, please see highlighting at yellow tab), in which the Office states:

The reference required by 37 CFR 1.78(a)(2) or (a)(5) must be included in an application data sheet (37 CFR 1/76), or the specification must contain, or be amended to contain, such reference in the first sentence following the title. Previously the Office indicated that if an applicant includes a benefit claim in the application but not in the manner specified by 37 CFR 1.78 (a) (e.g., if the claim

is included in an oath or declaration or the application transmittal letter) within the time period set forth in 37 CFR 1.78(a), the Office will not require a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) to correct the claim if the information concerning the claim was recognized by the Office as shown by its inclusion on the filing receipt. . . . The Office will continue to follow this practice. (emphasis added).

As discussed with the Examiner, Applicants respectfully submit that they have satisfied each of the above requirements for recognizing an improper priority claim without requiring either the petition set forth in 37 CFR 1.78(a) or the fee under 37 CFR 1.17(t). First, Applicants included a claim for priority within the time period specified by 37 CFR 1.78(a)(2) by including a request in the application transmittal letter submitted with the present application on October 1, 2003 to "[a]mend the specification by inserting before the first line the sentence . . . " setting forth the priority claim for this application (see Appendix 1 at yellow tab). Second, the filing receipt for this application lists each application, and its relationship to the other applications, to which Applicants attempted to claim priority in the transmittal letter (see Appendix 1 at yellow tab). Finally, Applicants have herein requested that the specification be amended to include the priority claim originally set forth in the application transmittal letter.

Hence, Applicants respectfully request that the Office accept Applicants' priority claim without requiring either the petition as set forth in 37 CFR 1.78(a)(2) or the fee as set forth in 37 CFR 1.17(t) and amend the specification as requested in this response and request for reconsideration.

Oath/Declaration Determination:

The Examiner has noted that the oath or declaration is defective because of non-initialed and/or non-dated alterations that have been made to the oath or declaration. Concurrent with submitting this response and request for reconsideration, Applicants have submitted a request to correct inventorship. The inventorship for this application no longer includes Dan Eaton and therefore, Applicants respectfully submit that this

objection is overcome. Applicants respectfully request that the Examiner withdraw this objection.

Double Patenting Determination:

The Examiner has provisionally rejected claims 22-26, 29 and 31 under the judicially created doctrine of obviousness-type double patenting due to claims 27-32 and 35-37 of U.S. Application No. 09/944,862. Applicants have allowed U.S. Application No. 09/944,862 to go abandoned and therefore, have overcome this rejection. Applicants respectfully request that the Examiner withdraw this rejection.

Rejection under 35 U.S.C. § 101:

Specific Utility

The Examiner rejected claims 22-34 under 35 U.S.C. § 101, alleging that the claimed invention is not supported by either a specific and substantial utility or a well established utility. Applicants respectfully disagree.

More specifically, the Examiner maintains that the ability to stimulate or inhibit lymphocyte proliferation in the MLR assay, which is an artificial *in vitro* system, does not provide for what specific conditions or for which specific diseases the claimed invention would predictably function. Thus, the Examiner alleges that Applicants' assertion of utility for the present invention is not specific. In particular, the Examiner rejects Applicant's assertion that the claimed invention could be useful for the treatment of conditions where enhancement or inhibition of the immune response would be beneficial because, according to the Examiner, there are many such conditions and it is not predictable in which conditions the claimed invention may function, if any.

Applicants respectfully disagree. "Specific utility" is defined as:

[a] utility that is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. For example, a polynucleotide whose use is disclosed simply as a 'gene probe' or

'chromosome marker' would not be considered to be *specific* in the absence of a disclosure of a specific DNA target. Similarly, a general statement of diagnostic utility, such as diagnosing an unspecified disease, would ordinarily be insufficient absent a disclosure of what condition can be diagnosed.

Revised Interim Utility Guidelines Training Materials, pgs. 5-6 (<http://www.uspto.gov/web/offices/pac/utility/utilityguide.pdf>).

According to this definition (found in the training materials), the present invention is supported by a "specific" utility. In particular, the utility of the present invention is specific to the subject matter claimed. That is, the claimed invention is a class of polypeptide molecules that shares at least 95% sequence identity with SEQ ID NO: 83 (or with the polypeptide encoded by the nucleic acid of SEQ ID NO: 82), and that has the characteristic of being capable of inhibiting T-cell proliferation. It is this property, the ability to inhibit T-cell proliferation, which provides one utility of the present invention. For example, the specification discloses at page 141, that "[c]ompounds which inhibit proliferation of lymphocytes are useful therapeutically where suppression of an immune response is beneficial." Thus, although Applicants have not named specific diseases in their application, Applicants have, as required by the definition of "specific" in the training materials, disclosed the condition to be treated by the sequence of the present invention, *i.e.* immune response.

Further, one of ordinary skill in the art would appreciate that only certain diseases involve treatment of the immune response. For example, those of ordinary skill in the art would appreciate that autoimmune diseases are diseases that involve treatment of the immune response. As support for this, Applicants respectfully direct the Examiner's attention to the attached Declaration of Sherman Fong, Ph.D (Appendix 2). In his declaration, Dr. Fong attests that it is his "considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in *autoimmune diseases*." (emphasis added).

For the foregoing reasons, the utility of being able to suppress immune response by inhibiting proliferation of T-cells is specific to the claimed sequences and therefore, Applicants respectfully request that the Examiner withdraw this rejection.

Mixed Lymphocyte Assay In the Present Application

As the Examiner notes, MLC is a special case of antigen stimulation in which T lymphocytes respond to foreign histocompatibility antigen on unrelated lymphocytes or monocytes. The Examiner also notes that the degree of reactivity observed in a MLC correlates with the degree of antigenic disparity between responding and stimulating cells. The Examiner's explanation of the MLC assay is very helpful in understanding the present invention. However, the Examiner recognizes the MLC assay as a general predictor of immune response, rather than as it is used in the present application. More specifically, in the present invention, the mixed lymphocyte assay is used as a measure of the ability of a particular test substance to stimulate or inhibit T-cell proliferation. Thus, it is useful to understand particular features of the assay as practiced in the present invention.

Hence, Applicants again respectfully direct the Examiner's attention to the attached Declaration of Sherman Fong, Ph.D. Dr. Fong explains at paragraph 6:

In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells.

At paragraph 7 of his declaration, Dr. Fong explains the significance of creating a sample comprising mainly dendritic cells:

Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naïve T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once

activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells.

Thus, as Dr. Fong explains at paragraph 8:

The MLR assay of the present application is designed to measure the ability of a test substance to “drive” the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

Similarly, the MLR assay of the present invention is useful for measuring the ability of a test substance (or “test sample” as it is referred to in the specification at page 141) to “inhibit” the drive of dendritic cells to induce the proliferation of T-cells. Therefore, as used in the present invention, the MLR assay is not a general predictor of immune response but rather is a specific assay designed to test the ability of a sample, such as the polypeptide of SEQ ID NO:83 or a polypeptide encoded by the nucleic acid of SEQ ID NO:82, to inhibit the drive of dendritic cells to induce T-cell proliferation.

MLR In Vitro Results Correlate With Observed In Vivo Results

In rejecting the present invention for lack of utility, the Examiner also cites three articles, Piccotti *et al.*, Campo *et al.* and Kahan. The Examiner argues that these articles support the argument that the claimed invention is not supported by a utility because the MLR (MLC) assay does not appear to be predictive of general immune responses *in vivo*. However, as mentioned above, the claims do not require predictability of general immune responses nor is the utility of the present invention dependent on the predictability of general immune responses.

Rather, the claims of the present invention are directed to polypeptides which inhibit stimulated T-cell proliferation. This characteristic of the claimed polypeptides is demonstrated by Example 34, which is a particular MLR assay designed to test the ability of a test sample, such as a polypeptide of the present invention, to inhibit T-cell proliferation, as described at page 141 of the specification. Thus, Applicants respectfully submit that the Examiner’s reliance on the above articles is misplaced.

In particular the Examiner relies on Kahan and argues that Kahan teaches that no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy because there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions. Applicants respectfully disagree that this teaching of Kahan would lead one of skill in the art to conclude that the present invention is not supported by either a specific and substantial or a well-established utility. The statement from Kahan that the Examiner relies on in rejecting the present claims is not particular to the MLR assay, but rather is a general statement intended to highlight difficulties in the development of efficacious immunosuppressive regimens.

Moreover, numerous *in vitro* MLR assay results which show either inhibition or stimulation of T cell proliferation by a test substance, as does the MLR in the present invention, have been confirmed *in vivo*. See Appendix 3. For example, in 1993, Wolos *et al.*, reported that *in vitro* MDL 28,842 (a S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5'-fluror-4',5'-didehydro-5'-deoxyadenosine) inhibited the generation of cytotoxic T-cells in MLR with an IC50 of less than 0.1 microM. Further, MDL 28,842 completely inhibited the generation cytotoxic T-cells when added up to 3 days after the initiation of culture with no apparent cell toxicity. *In vivo*, MDL 28,842 inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells. MDL 28,842 was also evaluated in the model of graft rejection. Skin allografts on animals given MDL 28,842 survived for 12.2 days, compared to 8.7 days for control animals. Wolos *et al.*, "Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8 (Appendix 3A).

Similarly, in 1995, Fung-Leung *et al.*, reported that tepoxalin suppressed murine T-cell proliferation in MLR and then demonstrated that oral administration of tepoxalin to mice suppressed local graft-versus host disease by about 40%. See Fung-Leung *et al.*, "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8 (Appendix 3B). Townsend *et al.*, reported in 1998, that a CD4-CDR3

peptide analog exhibited similar results both *in vitro* in MLR assays and *in vivo* in analyses of the effectiveness of the peptide analog at preventing graft-versus-host disease. Townsend *et al.*, "Combination therapy with a CD4-CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model." *Clin Immunol. Immunopathol.* 1998 86(1):115-9 (Appendix 3C). See also Townsend *et al.*, "Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier." *Blood.* 1996 88(8):3038-47 (Appendix 3D); Furukawa *et al.*, "Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro." *Clin Immunol. Immunopathol.* 1996 79(1):25-35 (Appendix 3E). Thus, the inhibition of T-cell proliferation by SEQ ID NO: 83 (or with the nucleic acid sequence encoding the polypeptide of SEQ ID NO: 82); as seen in the MLR results described at page 141 of the specification, would be expected to correlate with *in vivo* results.

MLR Measures the Ability of a Test Substance to Inhibit T-Cell Proliferation in the Present Application

Additionally the Examiner rejects the utility of the present claims because the Examiner argues that MLC is a measure of alloreactivity of one individual to another, rather than a general measure of immune function. While Applicants do not disagree with the Examiner's general conclusion of a utility of MLC, in the present invention, MLC (or MLR) is used to assess the ability of a *test sample* to inhibit or stimulate T-cell proliferation, as discussed above. Thus, the claims should not be rejected based on a general utility of a MLC assay.

Controls for MLR

The Examiner also asserts that the instant specification fails to provide sufficient detail of the assay performed. Specifically, the Examiner notes that there are several controls for MLR that are known in the art and are recognized as important for obtaining meaningful results for this assay.

As the Examiner recognizes, several important controls for MLR are known in the art. In addition, Applicants disclose, at page 141 of the specification that CD4-IgG may be used as a control in practicing the MLR assay in connection with the present invention. One of ordinary skill in the art would appreciate that CD4-IgG is an antibody that might be used as a negative control by blocking or preventing activation of allogeneic responder cells.

Additionally, Applicants disclose that cell culture media can be used as a control. Skilled artisans would appreciate that cell culture media would serve as a control by providing a measure of background levels. Applicants have also incorporated by reference the procedures described in Current Protocols in Immunology, unit 3.12. (See Exhibit 2A, Fong Declaration). Current Protocols teaches that "separate wells with control cultures should be set up that include – for each dose of responder and stimulator cells – replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells." Current Protocols also teaches that values obtained from these controls will reflect background proliferation levels. In addition, negative controls, such as wells with either only stimulator cells or only responder cells might be included as a parameter of the MLR assay. Thus, sufficient controls for MLR are both known in the art and disclosed by Applicants such that meaningful results can be achieved using this assay.

Interpretation of MLR Results

Lastly, the Examiner asserts that the specification fails to provide any data or evidence of the results of the assay and therefore alleges that one of skill in the art cannot evaluate the conclusion. More specifically, the Examiner contends that there is no indication of the statistical significance that must occur for determination of a positive result in the assay. However, the specification makes clear that any decrease below control is a positive result, with decreases of less than or equal to 80% being preferred. Further, Dr. Fong attests that it is "his considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, one of skill in the art would expect to find a

practical utility when an inhibition of the immune response is desired such as in autoimmune diseases.” Hence, Applicants have overcome the Examiner’s rejection of claims 25-34 for lack of utility under 35 U.S.C. § 101 and respectfully request that this rejection be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph:

Enablement

The Examiner contends that because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention.

Applicants respectfully disagree. As discussed above, the claimed polypeptide has the specific, substantial, and credible utility of encoding a polypeptide which inhibits the proliferation of stimulated T-lymphocytes as demonstrated in the MLR assay experiment discussed in Example 34 at page 141 of the application. Applicants respectfully request the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. § 112 ¶1 for alleged inadequate disclosure on how to use the claimed invention.

Wands Analysis

The Examiner also rejects claims 27-34 for lack of enablement, based on an analysis of the *Wands* factors. Applicants respectfully disagree with the Examiner’s analysis and conclusion.

1. The Nature of the Invention

The first *Wands* factor analyzed is the nature of the invention. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). As the Examiner notes, the claimed invention is a polypeptide having at least 95% sequence identity to SEQ ID NO: 83, or the extracellular domain thereof, with or without a signal peptide, or the transmembrane domain of SEQ ID NO: 83, or polypeptides having sequence homology thereto, or

chimeric polypeptides thereof. The claimed invention is also defined to encompass only those polypeptide capable of inhibiting proliferation of stimulated T-lymphocytes.

2. The State of the Prior Art

The second *Wands* factor is the state of the prior art. The Examiner contends that the state of the prior art regarding the ability of a protein to inhibit the MLR assay is "unpredictable and uncertain, since it is an artificial *in vitro* system and does not indicate for what specific conditions and for which specific diseases the protein would be useful."

Applicants respectfully disagree. As discussed above, MLR is a well-established assay and in the present invention this assay is used for assessing the ability of a test compound to stimulate or inhibit T cell proliferation. Also as discussed above, the MLR assay is described in standard textbooks, including *Current Protocols in Immunology*, and in numerous journal articles.

3. Relative Skill of Those in the Art

The level of skill in the art is high.

4. Level of Predictability in the Art

The art of the claimed subject matter is predictable. According to MPEP 2164.01(b), "the 'predictability or lack thereof' in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention." As discussed above, numerous *in vitro* MLR assay results which show either inhibition or stimulation of T cell proliferation by a test substance, as does the MLR in the present invention, have been confirmed *in vivo*. See Appendix 3. See also Wolos *et al.*, "Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of *in vitro* and *in vivo* allogeneic responses." *Cell Immunol.* 1993 149(2):402-8; Fung-Leung *et al.*, "Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection." *Transplantation.* 1995 60(4):362-8; Townsend *et al.*, "Combination therapy with a CD4-

CDR3 peptide analog and cyclosporine A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplant model.” *Clin Immunol. Immunopathol.* 1998 86(1):115-9; Townsend *et al.*, “Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier.” *Blood.* 1996 88(8):3038-47; Furukawa *et al.*, “Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro.” *Clin Immunol. Immunopathol.* 1996 79(1):25-35.

5. Presence of Working Examples

Wands also requires the Examiner to consider the presence or absence of working examples. *Wands* 858 F.2d at 737. As the Examiner notes, Applicant's describe the working MLR assay example at page 141 of the specification.

The Examiner contends that there is no correlation taught or well known in the art between the MLR assay and *in vivo* treatment of diseases involving the immune response. Applicants respectfully disagree. As mentioned above, numerous *in vitro* MLR assay results which show either inhibition or stimulation of T cell proliferation by a test substance, as does the MLR in the present invention, have been confirmed *in vivo*. Further, one such example is discussed in paragraph 9 of Dr. Fong's declaration:

For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Guber *et al.* PNAS 88, 4143 (1991)(Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson *et al.* Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)].

Thus, Applicants respectfully submit by describing the MLR assay at page 131, Applicants have disclosed sufficient working examples.

6. Breadth of the Claims

The claims are not overly broad but instead, are directed to polypeptides with at least 95% sequence identity to SEQ ID NO: 83, or the extracellular domain thereof, with or without a signal peptide, or the transmembrane domain of SEQ ID NO: 83, or polypeptides having sequence homology thereto, or chimeric polypeptides thereof.

7. Amount of Direction or Guidance

Finally, the Examiner asserts that there is no guidance in the specification regarding, for example, how patients would be treated with the disclosed polynucleotides or how the compounds would be administered. The claims, however, do not require that the polynucleotide be used to treat a patient. One of skill in the art will know how to use the disclosed polynucleotides in connection with the MLR assay. The results of the assay alone provide one of skill in the art with valuable information to use in treating various immune response conditions. One example of a condition that could be treated based on information obtained in the MLR assay is graft versus host disease. In particular, information gathered about the ability of a particular polypeptide to inhibit T-cell proliferation would enable one skilled in the art to predict the success of a graft and adjust the aggressiveness of immunosuppressive therapy.

8. Quantity of Experimentation

The *Wands* factors are analyzed as a tool to determine whether undue experimentation is required to practice a claimed invention. However, the Federal Circuit has stated that:

[t]he determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art (citations omitted). The test is not merely quantitative, since a *considerable amount of experimentation is permissible*, if it is merely routine, or *if the specification provides a reasonable amount of guidance* with respect to the direction in which the experimentation should proceed. See *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

Given the established utility of a MLR assay as used in the present invention, the level of skill in the art, and the significant disclosure found in the specification, any experimentation that might be required to practice the claimed invention would be routine and not undue. Therefore, Applicants submit that the present invention is enabled by the specification and respectfully request that the Examiner withdraw this ground of rejection.

CONCLUSION

Applicants believe this Request for Reconsideration fully responds to the Office Action. Applicants respectfully request the Examiner grant early allowance of this application. The Examiner is invited to contact the undersigned attorney for the Applicant via telephone if such communication would expedite this application.

Respectfully submitted,



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United States Patent and Trademark Office OG Notices: 18 March 2003

Claiming the Benefit of a Prior-Filed Application under
35 U.S.C. 119(e), 120, 121, and 365(c)

Summary: This notice clarifies how benefit claims under 35 U.S.C. 119(e), 120, 121 and 365(c) must be presented in applications in order to be in compliance with the relevant statute and patent regulations, and accepted by the United States Patent and Trademark Office (Office).

35 U.S.C. 120 Benefit Claims

Benefit claims under 35 U.S.C. 120 must include a specific reference to the earlier filed (nonprovisional) application for which a benefit is sought. A "specific reference" requires: (1) the identification of the prior (nonprovisional) application by application number; and (2) an indication of the relationship between the nonprovisional applications, except for the benefit claim to the prior application in a continued prosecution application (CPA). The relationship between any two nonprovisional applications will be an indication that the later-filed nonprovisional application is either a continuation, divisional, or continuation-in-part of the prior-filed nonprovisional application. When there are benefit claims to multiple prior nonprovisional applications (e.g., a string of prior nonprovisional applications), the relationship must include an identification of each nonprovisional application as either a continuation, divisional, or continuation-in-part application of a specific prior nonprovisional application for which a benefit is claimed. The identification is needed in order to be able to verify if copendency exists throughout the entire chain of prior nonprovisional applications.

35 U.S.C. 119(e) Benefit Claims

Benefit claims under 35 U.S.C. 119(e) must include a specific reference to the earlier filed provisional application for which a benefit is sought. A specific reference in this situation, however, only requires identification of the prior provisional application by the application number. No relationship between the subject nonprovisional application and the prior provisional application should be specified. If the subject nonprovisional application was not filed within twelve months of the filing date of the provisional application, the subject application must also include a benefit claim under 35 U.S.C. 120 to an intermediate prior nonprovisional application that was filed within twelve months of the filing date of the provisional application. Therefore, in addition to the identification of the provisional application, the proper benefit claim for this situation must also identify the intermediate prior nonprovisional application that is directly claiming the benefit of the provisional application, and indicate the relationship between the nonprovisional applications (e.g., an indication that the subject application is a continuation of the intermediate prior nonprovisional application).

Statement of the Problem

Background: Some applicants have been submitting patent applications which include, or are amended to include, at the beginning of the specification, a statement that benefits are claimed under 35 U.S.C. 119(e) and/or 120 to prior applications, followed by a listing of many prior nonprovisional and provisional applications. The listings do not indicate: (1) the specific relationship (i.e., continuation, divisional, or continuation-in-part) between the nonprovisional applications, as required by 37 CFR 1.78(a)(2)(i); and/or (2) each nonprovisional application which is directly claiming priority to a provisional application. Without such information, the Office does not have sufficient information to enter the benefit claims into the Office's computer database.

When entering benefit claims for an application into the Office's database, the relationship (i.e., continuation, divisional or continuation-in-part) between the nonprovisional applications is required. Further, the system will not accept any benefit claim to a provisional application if the provisional application was filed more than twelve months prior to the filing date of the subject application, unless the applicant clearly identifies, and claims the benefit of, a prior nonprovisional application that was filed within twelve months of the filing date of the provisional application. Accordingly, if benefit claims are presented without all the required information, the Office will not be able to enter such benefit claims into the Office's database, the filing receipt will not reflect the prior application(s), and the projected publication date will not be calculated as a function of an earlier application's filing date.

The specific relationships between (each of) the nonprovisional applications in a chain of nonprovisional applications are also important because such information will be printed in the application publication, and/or patent. Furthermore, the designation of an application as a continuation (rather than as a continuation-in-part) is an indication that the entire invention claimed in an application has support in the prior application, whereas the designation of an application as a continuation-in-part is an indication that the claimed invention is not entirely supported by the prior application. Thus, the specific relationship between nonprovisional applications in a chain of benefit claims, and the indication of the specific nonprovisional application(s) that is directly claiming the benefit of a provisional application, will provide the information that is needed by examiners and the public in order to determine the effective prior art date of the application publication, or patent, under 35 U.S.C. 102(e).

When benefit claims are required to, but do not, include: (1) an identification of (all) intermediate benefit claims, and/or (2) the relationship between nonprovisional applications, the Office may not be able to publish applications promptly after the expiration of a period of eighteen months from the earliest filing date for which a benefit is sought under title 35, United States Code (eighteen-month publication), nor have the accuracy desired of such benefit claims in application publications. Further, the objection (by the Office), correction (by applicant), and review/entry of changes (by the Office) cycle for non-compliant benefit claims is a burdensome effort on both applicants and the Office that can be totally avoided if such benefit claims are properly submitted the first time. Accordingly, it is hoped that applicants will submit benefit claims with all the required information as set forth in this notice and, correspondingly, avoid submitting non-compliant benefit claims that leads to extra work for both the Office and applicants.

Procedures for Making Proper Benefit Claims

Part I: Reference to Prior Nonprovisional Application(s)
 Per 37 CFR 1.78(a)(2)(i) Must Include Identification of, and
 Relationship Between, Applications

35 U.S.C. 120 provides that no application shall be entitled to the benefit of an earlier filed application unless it contains, or is amended to contain, a specific reference to the earlier filed application. The specific reference required by 35 U.S.C. 120 is the reference required by 37 CFR 1.78(a)(2). 37 CFR 1.78(a)(2)(i) requires that any nonprovisional application that claims the benefit of one or more prior-filed copending nonprovisional applications, or international applications designating the United States, must contain, or be amended to contain, a reference to each such prior-filed application, identifying it by application number (consisting of the series code and serial number), or international application number and international filing date, and indicating the relationship of the applications. 37 CFR 1.78(a)(2)(iv) also provides that a request for a continued prosecution application (CPA) under 37 CFR 1.53(d) is the specific reference required under 35 U.S.C. 120 to the prior-filed application. Therefore, except for the benefit claim to the prior-filed application in a CPA, benefit claims under 35 U.S.C. 120, including claims under 35 U.S.C. 121 and 365(c), must not only identify the earlier application by application number, or by international application number and international filing date, but they must also indicate the relationship between the applications.

Examples

The relationship between the applications is whether the subject application is a continuation, divisional, or continuation-in-part of a prior-filed nonprovisional application. An example of a proper benefit claim is: "This application is a continuation of Application No. 10/ - , filed - ." A benefit claim that merely states: "This application claims the benefit of Application No. 10/ - , filed - ." does not comply with 37 CFR 1.78(a)(2)(i), since the relationship between the applications is not stated. In addition, a benefit claim that merely states: "This application is a continuing application of Application No. 10/ - , filed - ." does not comply with 37 CFR 1.78(a)(2)(i) since the proper relationship, which includes the type of continuing application, is not stated. It is also noted that the status of each nonprovisional parent application (if it is patented or abandoned) should also be indicated, following the filing date of the parent nonprovisional application. An example of a proper benefit claim of a prior national stage of an international application is "This application is a continuation of U.S. Application No. X, which is the National Stage of International Application No. PCT/US - / - , filed - ." For additional examples of proper benefit claims, see Manual of Patent Examining Procedure (8th ed., August 2001) (MPEP), Section 201.11, Reference to First Application. Section 201.11 of the MPEP will be revised in the upcoming revision to reflect the clarification made in this notice about the required manner of making proper claims for the benefit of prior nonprovisional and provisional applications.

As stated previously, to specify the relationship between the nonprovisional applications, applicant must specify whether the subject application is a continuation, divisional, or continuation-in-part of the prior nonprovisional application. Note that the terms are exclusive. An application cannot be, for example, both a continuation

and a divisional, or a continuation and a continuation-in-part, of the same parent application. Moreover, if the benefit of more than one nonprovisional parent application is claimed, the relationship must include an identification of each nonprovisional application as a continuation, divisional, or continuation-in-part application of the immediate prior nonprovisional application for which a benefit is claimed in order to establish co-pendency throughout the entire chain of prior-filed parent nonprovisional applications. For example, the following two statements are improper: "This application claims the benefit of Application Nos. C, B, and A." and "This application is a continuing application of Application Nos. C, B, and A." On the other hand, the following statement is proper and acceptable: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which is a continuation of Application No. A, filed - ."

Sanctions for Making Improper Benefit Claims to Nonprovisional Applications

Any benefit claim under 35 U.S.C. 120, 121 or 365(c) that does not identify a prior application and also specify a relationship between each of the applications will not be in compliance with 37 CFR 1.78(a)(2)(i), and will not be considered to contain a specific reference to a prior application as required by 35 U.S.C. 120. Such a benefit claim will not be recognized by the Office and will not be included on the filing receipt for the application, even if the claim appears in the first sentence of the specification or an application data sheet (37 CFR 1.76), because the Office does not have sufficient information to enter the benefit claim into the Office's database. As a result, publication of the application will not be scheduled as a function of the prior application's filing date. The Office plans to notify applicants on, or with, the filing receipt that a benefit claim may not have been recognized because it did not include the proper reference. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided to the applicant shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a)(2)(ii) and thus avoid the need to submit a petition under 37 CFR 1.78(a)(3) and the surcharge set forth in 37 CFR 1.17(t). Failure to timely submit the reference required by 37 CFR 1.78(a)(2)(i) is considered a waiver of any benefit claim under 35 U.S.C. 120, 121 or 365(c) unless a petition to accept an unintentionally delayed claim under 37 CFR 1.78(a)(3), the surcharge set forth in 37 CFR 1.17(t), and the required reference, including the relationship of the applications (unless previously submitted) are filed. For example, if a benefit claim is submitted without the specific relationship between the nonprovisional applications before the expiration of the period, and the specific relationship between the nonprovisional applications is subsequently submitted after the expiration of the period, a petition and the surcharge would be required.

Part II: Reference to Prior Provisional Application(s) Per
37 CFR 1.78(a)(5)(i) Should Only Include Identification of Prior
Provisional Application(s)

When the domestic benefit of a prior provisional application is being claimed under 35 U.S.C. 119(e), however, the relationship between the two applications should not be specified. 35

U.S.C. 119(e) provides that a nonprovisional application claiming the benefit of a provisional application must be filed within twelve months of the provisional application and must contain, or be amended to contain, a specific reference to the provisional application. The specific reference required by 35 U.S.C. 119(e) is the reference required by 37 CFR 1.78(a)(5). 37 CFR 1.78(a)(5)(i) requires that any nonprovisional application, or international application designating the United States, claiming the benefit of one or more prior-filed provisional applications must contain, or be amended to contain, a reference to each such prior-filed provisional application identifying it by provisional application number. No relationship should be specified whenever a claim for the benefit of a provisional application under 35 U.S.C. 119(e) is made.

If a relationship between a nonprovisional application and a prior provisional application is submitted, however, it may be unclear whether applicant wishes to claim the domestic benefit of the provisional application under 35 U.S.C. 119(e), or the benefit of an earlier application's filing date under 35 U.S.C. 120. Thus, applicants seeking to claim the domestic benefit of a provisional application under 35 U.S.C. 119(e) should not state that the application is a "continuation" of a provisional application, nor should it be stated that the application claims benefit under 35 U.S.C.

120 of a provisional application. If such a claim is submitted in an application transmitted to the Office other than through the Electronic Filing System, it will be entered into the Office computer system as a claim to the "benefit" of the provisional application. Although 35 U.S.C. 120 does not preclude a benefit claim to a provisional application (that is, one could obtain the benefit under 35 U.S.C. 120 of a prior filed provisional application), such a benefit claim under 35 U.S.C. 120 is not recommended as such a claim may have the effect of reducing the patent term, as the term of a patent issuing from such an application may be measured from the filing date of the provisional application pursuant to 35 U.S.C. 154(a)(2). Instead, applicants should state "This application claims the benefit of U.S. Provisional Application No. 60/ - , filed - ", or "This application claims the benefit of U.S. Provisional Application No. 60/ - , filed - , and U.S. Provisional Application 60/ - , filed - ." See MPEP 201.11, Reference to First Application (8th ed., August 2001).

Part III: If Benefit is Claimed of a Prior Provisional Application Which was Filed More Than One Year Before the Subject Application, Then Each Prior Nonprovisional Application(s) Claiming Benefit of the Provisional Must be Specified

Any nonprovisional application that directly claims the benefit of a provisional application under 35 U.S.C. 119(e) must be filed within twelve months from the filing date of the provisional application. As noted above, an application that itself directly claims the benefit of a provisional application should identify, but not specify the relationship to, the provisional application. If the subject nonprovisional application is not filed within the twelve month period, however, it still may claim the benefit of the provisional application via an intermediate nonprovisional application under 35 U.S.C. 120. The intermediate nonprovisional application must have been filed within twelve months from the filing date of the provisional application and the intermediate nonprovisional application must have claimed the benefit of the provisional application. Further, it must be clearly indicated that the intermediate nonprovisional application is

claiming the benefit under 35 U.S.C. 119(e) of the provisional application. This identification of the intermediate nonprovisional application is necessary so that the Office can determine whether the intermediate nonprovisional application was filed within twelve months of the filing date of the provisional application, and thus, whether the benefit claim is proper.

Examples

Applicant should state such a benefit claim as follows: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which claims the benefit of U.S. Provisional Application No. A, filed - ." A benefit claim that merely states "This application claims the benefit of nonprovisional Application No. C, filed - , nonprovisional Application No. B, filed - , and provisional application No. A, filed - " would be improper where the subject application was not filed within twelve months of the provisional application.

Where the benefit of more than one provisional application is being claimed, the intermediate nonprovisional application(s) claiming the benefit of each provisional application must be clearly indicated. Applicant should state, for example, "This application is continuation of Application No. D, filed - , which is a continuation-in-part of Application No. C, filed - , Application No. D claims the benefit of U.S. Provisional Application No. B, filed - , and Application No. C claims the benefit of U.S. Provisional Application No. A, filed - ." An example of a proper benefit claim of a prior national stage of an international application, which claims the priority to a provisional application, is "This application is a continuation of U.S. Application No. Y, which is the National Stage of International Application No. PCT/US - / - , filed - , which claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application X, filed - ."

Sanctions for Making Improper Benefit Claims to Provisional Applications

If a benefit claim to a provisional application is submitted without an indication that an intermediate nonprovisional application directly claims the benefit of the provisional application and the instant nonprovisional application is not filed within the twelve month period, or the relationship between nonprovisional applications is not indicated, the Office will not have sufficient information to enter the benefit claim into the computer database. Therefore, the Office will not recognize such a benefit claim, and will not include the benefit claim on the filing receipt. The Office plans to notify applicants on, or with, the filing receipt that a benefit claim may not have been recognized because information regarding the intermediate nonprovisional application(s) and/or the relationship between each nonprovisional application have not been provided. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided to the applicant shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a) and thus avoid the need to submit a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t). Failure to timely submit the reference required by 37 CFR 1.78(a) is considered a waiver of any benefit claim under 35 U.S.C. 119(e),

- 120, 121 or 365(c) unless a petition under 37 CFR 1.78(a), the surcharge set forth in 37 CFR 1.17(t), identification of the intermediate nonprovisional application which claims the benefit to the provisional application, and the relationship between each nonprovisional application are filed.

Part IV: Office Practice to Not Require Petition and Surcharge if Benefit Claim is Not Present in the Proper Place But is Recognized By Office Continues But Applicants Are Advised That Proper Reference Must be Presented

The reference required by 37 CFR 1.78(a)(2) or (a)(5) must be included in an application data sheet (37 CFR 1.76), or the specification must contain, or be amended to contain, such reference in the first sentence following the title. Previously, the Office indicated that if an applicant includes a benefit claim in the application but not in the manner specified by 37 CFR 1.78(a) (e.g., if the claim is included in an oath or declaration or the application transmittal letter) within the time period set forth in 37 CFR 1.78(a), the Office will not require a petition under 37 CFR 1.78(a) and the surcharge under 37 CFR 1.17(t) to correct the claim if the information concerning the claim was recognized by the Office as shown by its inclusion on the filing receipt. If, however, a claim is included elsewhere in the application and not recognized by the Office as shown by its absence on the filing receipt, the Office will require a petition and the surcharge to correct the claim. See Requirements for Claiming the Benefit of Prior-Filed Applications Under Eighteen-Month Publication Provisions, 66 Fed. Reg. 67087, 67089-90 (Dec. 28, 2001). The Office will continue to follow this practice.

Sanctions for Making Improper Benefit Claims

Applicants are simply being advised by this notice that the Office will not recognize any benefit claim where there is no indication of the relationship between the nonprovisional applications, or no indication of the intermediate nonprovisional application that is directly claiming the benefit of a provisional application. Applicants are also reminded that, even if the Office has recognized a benefit claim that includes the proper reference by entering it into the Office's database and including it on applicant's filing receipt, the benefit claim is not a proper benefit claim under 35 U.S.C. 119(e) and/or 35 U.S.C. 120, and 37 CFR 1.78, unless the reference is included in an application data sheet, or the first sentence of the specification, and all other requirements are met.

Part V: Correcting or Adding a Benefit Claim After Filing

The Office will not grant a request for a corrected filing receipt to include a benefit claim unless a proper reference to the prior application(s) is included in the first sentence of the specification, or an application data sheet, within the time period required by 37 CFR 1.78(a). Any request for corrected filing receipt to include a corrected or added benefit claim must be submitted within the time period required by 37 CFR 1.78(a) and be accompanied by an amendment to the specification, or an application data sheet. If the proper reference was previously submitted, a copy of the amendment, the first page of the specification, or the application data sheet, containing the claim should be included with the request for corrected filing receipt. The Office plans to notify applicants on, or with, the

filing receipt that a benefit claim may not have been recognized because it did not include the proper reference. Applicants are advised that only the benefit claims that are listed on the filing receipt have been recognized by the Office. Since the filing receipt and the notification will usually be provided shortly after the filing of the application, applicants should have sufficient opportunity to submit the proper benefit claims within the time period set in 37 CFR 1.78(a) and thus avoid the need to submit a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t). Therefore, applicants should carefully and promptly review their filing receipts in order to avoid the need for a petition and the surcharge.

When an unintentionally delayed benefit claim is submitted with a petition under 37 CFR 1.78(a) and the surcharge set forth in 37 CFR 1.17(t), the benefit claim must include a proper reference to the prior application(s) in order for the petition to be granted. The reference to the prior application(s) must include: (1) the relationship between nonprovisional applications (i.e., continuation, divisional, or continuation-in-part), and (2) the indication of any intermediate application that is directly claiming the benefit of a provisional application, in order to establish copendency throughout the entire chain of prior applications.

Applicants are also reminded that, if an amendment to the specification, or an application data sheet (ADS), is submitted in an application under final rejection, the amendment or ADS must be in compliance with 37 CFR 1.116. The amendment or ADS filed in an application under final rejection will not be entered as a matter of right. See MPEP 714.12 and 714.13. Therefore, applicants should consider filing a request for continued examination (RCE) (including fee and submission) under 37 CFR 1.114 with the petition to accept an unintentionally delayed benefit claim, the surcharge, and an amendment that adds the proper reference to the first sentence of the specification or an ADS.

Part VI: Each Intermediate Prior Application Must Have Proper Reference

If the benefit of more than one prior application is claimed, applicant should also make sure that the proper references are made in each intermediate nonprovisional application in the chain of prior applications. If an applicant desires, for example, the following benefit claim: "This application is a continuation of Application No. C, filed - , which is a continuation of Application No. B, filed - , which claims the benefit of U.S. Provisional Application No. A, filed - ," then Application No. C must include a benefit claim containing a reference to Application No. B and provisional Application No. A, and Application No. B must include a benefit claim containing a reference to provisional Application No. A.

Part VII: Adding an Incorporation-By-Reference Statement in a Benefit Claim is Not Permitted After Filing

An incorporation-by-reference statement added after the filing date of an application is not permitted because no new matter can be added to an application after its filing date. See 35 U.S.C. 132(a). If an incorporation-by-reference statement is included in an amendment to the specification to add a benefit claim after the filing date of the application, the amendment would not be proper. When a benefit claim is submitted after the filing of an application, the reference to the prior application cannot include an incorporation-by-reference statement of the prior application. See *Dart Industries v. Banner*, 636 F.2d 684,

207 USPQ 273 (C.A.D.C. 1980). Therefore, the Office will not grant a petition to accept a benefit claim that includes an incorporation-by-reference statement of a prior application, unless the incorporation-by-reference statement was submitted on filing of the application.

Inquiries regarding this notice should be directed to Eugenia A. Jones or Joni Y. Chang, Legal Advisors, Office of Patent Legal Administration, by telephone at (703) 305-1622.

February 24, 2003

STEPHEN G. KUNIN
Deputy Commissioner for
Patent Examination Policy

"Express Mail" mailing label number EV 314842857 US

Date of Deposit 1 October 2003

REQUEST FOR FILING A CONTINUING PATENT APPLICATION UNDER 37 CFR § 1.53(b)(1)

Case No.	ANTICIPATED CLASSIFICATION OF THIS APPLICATION		PRIOR APPLICATION EXAMINER	ART UNIT
10466/484	CLASS	SUBCLASS	Nancy T. Vogel	1636

Address to:

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR § 1.53(b)(1), of pending prior application number 09/944,862, filed on August 31, 2001, entitled SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME.

- ☒ Copy Of the Prior application, including 34 sheets of drawings, 152 pages of Application
- ☒ Copy of the Declaration filed in the Prior application.
- ☐ PTO Form 1449 and Information Disclosure Statement.

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS (37 CFR 1.16(c))	20 - 20 =	0	x \$ 18 =	\$
	INDEPENDENT CLAIMS (37 CFR 1.16(b))	3 - 3 =	0	x \$ 84 =	\$
	MULTIPLE DEPENDENT CLAIMS (if applicable) (37 CFR 1.16(d))			+ \$280 =	\$ 0
				BASIC FEE (37 CFR 1.16(a))	\$ 770
				Total of above Calculations =	\$
	Reduction by 50% for filing by small entity (Note 37 CFR 1.9, 1.27, 1.28)				\$
				TOTAL =	\$770.00

- ☐ A verified statement to establish small entity status under 37 CFR 1.9 and 1.27
☐ is enclosed.
☐ was filed in prior application number _____ and such status is still proper and desired (37 CFR 1.28(a)).
- ☒ The Commissioner is hereby authorized to charge any fees which may be required under 37 CFR 1.16 and 1.17, or credit any overpayment to Deposit Account No. 23-1925. A duplicate copy of this sheet is enclosed.
- ☒ Enclosed is a check for \$ 770.00 to cover the filing fees.
- ☒ Cancel in this application original claims 1-21 of the prior application and otherwise enter the attached preliminary amendment before calculating the filing fee.
- ☒ The inventor(s) of the invention being claimed in this application is(are): Kevin P. Baker, David Botstein, Dan L. Eaton, Napoleone Ferrara, Ellen Filvaroff, Mary E. Gerritsen, Audrey Goddard, Paul J. Godowski, J. Christopher Grimaldi, Austin I. Gurney, Kenneth J. Hillan, Ivar J. Kljavin, Mary A. Napier, Margaret Ann Roy, Daniel Tumas, William I. Wood.
- ☐ This application is being filed by less than all the inventors named in the prior application. In accordance with 37 CFR 1.63(d)(2), the Commissioner is requested to delete the name(s) of the following person or persons who are not inventors of the invention being claimed in this application: _____.

10. ☒ Amend the specification by inserting before the first line the sentence: "This application is a ☒ continuation ☐ division of application number 09/944,862, filed This application is a continuation of, and claims priority under 35 USC Section 120 to, US Application 09/866,028 filed 5/25/2001, which is a continuation of, and claims priority under 35 USC Section 120 to, PCT Application PCT/US99/28301 filed 12/1/1999, which is a continuation-in-part of, and claims priority under 35 USC Section 120 to, US Application 09/254,311 file 3/3/1999, now abandoned, which is the National Stage filed under 35 USC Section 371 of PCT Application PCT/US98/25108 filed 12/1/1998, which claims priority under 35 USC Section 119 to US Provisional Application 60/075,945 filed 2/25/1998."
11. ☐ New formal drawings are enclosed.
12. ☐ Priority of foreign application number _____, filed on _____ respectively in _____ is claimed under 35 U.S.C. 119.
- ☐ The certified copy has been filed in prior application number _____, filed
13. ☒ A preliminary amendment is enclosed.
14. ☒ The prior application is assigned of record to GENENTECH, INC.
15. ☒ Also enclosed: Request To Use Computer Readable Form Of Sequence Listing From Parent Application Pursuant to 37 C.F.R. Section 1.821(e); and Copy of Sequence Listing (72) pages.
16. ☒ The power of attorney in the prior application is to: K. Shannon Mrksich, Reg. No. 36,675 and other attorneys at the firm of BRINKS HOFER GILSON & LIONE.
- a. ☒ The power appears in the original papers in the prior application.
- b. ☐ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
- c. ☒ Address all future correspondence to: (may only be completed by applicant, or attorney or agent of record.)

K. Shannon Mrksich
BRINKS HOFER GILSON & LIONE
P.O. BOX 10395
CHICAGO, IL 60610
(312)321-4200

10-1-03
Date

S. Mrksich
Signature

Name: K. Shannon Mrksich
Reg. No. 36,675

- ☐ Inventor(s)
☐ Assignee of complete interest
☒ Attorney or agent of record
☐ Filed under 37 CFR 1.34(a)
Registration Number if acting under 37 CFR 1.34(a): _____



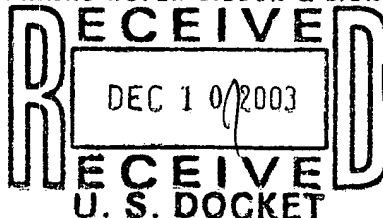
UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
 United States Patent and Trademark Office
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 P.O. Box 1450
 Alexandria, Virginia 22313-1450
 www.uspto.gov

APPL NO.	FILING OR 371 (c) DATE	ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	DRAWINGS	TOT CLMS	IND CLMS
10/677,471	10/02/2003	1636	770	10466/484	34	13	3

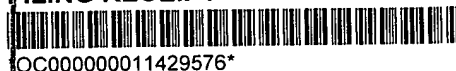
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 CHICAGO, IL 60610

BRINKS HOFER GILSON & LIONE



CONFIRMATION NO. 1021

FILING RECEIPT



OC000000011429576*

Date Mailed: 12/05/2003

Receipt is acknowledged of this regular Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Kevin P. Baker, Darnestown, MD;
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 Dan L. Eaton, San Rafael, CA;
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 William I. Wood, Hillsborough, CA;

Assignment For Published Patent Application

GENENTECH, INC.;

Domestic Priority data as claimed by applicant

This application is a CON of 09/944,862 08/31/2001
 and is a CON of 09/866,028 05/25/2001 PAT 6,642,360
 which is a CON of PCT/US99/28301 12/01/1999
 which is a CIP of 09/254,311 03/03/1999 ABN
 which is a 371 of PCT/US98/25108 12/01/1998

which claims benefit of 60/075,945 02/25/1998

Foreign Applications

If Required, Foreign Filing License Granted: 12/05/2003

Projected Publication Date: 03/18/2004

Non-Publication Request: No

Early Publication Request: No

Title

Secreted and transmembrane polypeptides and nucleic acids encoding the same

Preliminary Class

514

**LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15**

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Office of Export Administration, Department of Commerce (15 CFR 370.10 (j)); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:

Docket No.:

Serial No.:

Group Art Unit:

Filing Date:

Examiner:

For:

DECLARATION OF SHERMAN FONG, Ph.D. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Sherman Fong, Ph.D. declare and say as follows: -

1. I was awarded a Ph.D. in Microbiology by the University of California at Davis, CA in 1975.
2. After postdoctoral training and holding various research positions at Scripps Clinic and Research Foundation, La Jolla, CA, I joined Genentech, Inc., South San Francisco, CA in 1987. I am currently a Senior Scientist at the Department of Immunology/Discovery Research of Genentech, Inc.
3. My scientific Curriculum Vitae is attached to and forms part of this Declaration.
4. I am familiar with the Mixed Lymphocyte Reaction (MLR) assay, which has been used by me and others under my supervision, to test the immune stimulatory or immune inhibitory activity of novel polypeptides discovered in Genentech's Secreted Protein Discovery Initiative project.
5. The MLR assay is a well known and widely used proliferative assay of T-cell function, the basic protocols of which are described, for example, in Current Protocols in Immunology Vol. 1, Richard Coico, Series Ed., John Wiley & Sons, Inc., 1991, Unit 3.12. (Exhibit A). This publication is incorporated by reference in the description of the MLR protocol in the present application.

6. The T-lymphocytes or "T-cells" of our immune system can be induced to proliferate by a variety of agents. The MLR assay is designed to study a particularly important induction mechanism whereby responsive T-cells are cultured together (or "mixed"), with other lymphocytes that are "allogeneic", e.g. lymphocytes that are taken from different individuals of the same species. In the MLR protocol of the present application, a suspension of PBMCs that includes responder T-cells, is cultured with allogeneic PBMCs that predominantly contain dendritic cells. According to the protocol, the allogeneic "stimulator" PBMCs are irradiated at a dose of 3000 Rad. This irradiation is done in order to create a sample of cells that has mainly dendritic cells. It is known that the dendritic cell population among the PBMCs are differentially affected by irradiation. At low doses (500-1000 Rad), the proliferation of most cells, including the B cells in the PBMCs, is preserved, however, at doses above 2000 Rad, this function of B cells is abolished. Dendritic cells on the other hand, maintain their antigen presentation function even at a 3000 Rad dose of radiation. (See, e.g. Current Protocols in Immunology, *supra*, at 3.12.9). Accordingly, under the conditions of the MLR assay used to test the PRO polypeptides of the present invention, the stimulator PBMCs remaining after irradiation are essentially dendritic cells.
7. Dendritic cells are the most potent antigen-presenting cells, which are able to "prime" naive T cells *in vivo*. They carry on their surface high levels of major histocompatibility complex (MHC) products, the primary antigens for stimulating T-cell proliferation. Dendritic cells provide the T-cells with potent and needed accessory or costimulatory substances, in addition to giving them the T-cell maturing antigenic signal to begin proliferation and carry out their function. Once activated by dendritic cells, the T-cells are capable of interacting with other antigen presenting B cells and macrophages to produce additional immune responses from these cells. For further details about the properties and role of dendritic cells in immune-based therapies see, e.g. Steinman, Drug News Perspect. 13(10):581-586 (Exhibit B).
8. The MLR assay of the present application is designed to measure the ability of a test substance to "drive" the dendritic cells to induce the proliferation of T-cells that are activated, or co-stimulated in the MLR, and thus identifies immune stimulants that can boost the immune system to respond to a particular antigen that may not have been immunologically active previously.

9. Such immune stimulants find important clinical applications. For example, IL-12 is a known immune stimulant, which has been shown to stimulate T-cell proliferation in the MLR assay. IL-12 was first identified in just such an MLR [Gubler et al. PNAS 88, 4143 (1991) (Exhibit C)]. In a recent cancer vaccine trial, researchers from the University of Chicago and Genetics Institute (Cambridge, MA) have demonstrated the efficacy of the approach, relying on the immune stimulatory activity of IL-12, for the treatment of melanoma. [Peterson et al. Journal of Clinical Oncology 21 (12). 2342-48 (2003) (Exhibit D)] They extracted circulating white blood cells carrying one or more markers of melanoma cells, isolated the antigen, and returned them to the patients. Normally patients would not have an immune response to his or her own human antigens. The patients were then treated with different doses of IL-12, an immune stimulant capable of inducing the proliferation of T cells that have been co-stimulated by dendritic cells. Due to the immune stimulatory effect of IL-12, the treatment provided superior results in comparison to earlier work, where patients' own dendritic cells were prepared from peripheral blood mononuclear cells (PBMCs), treated with antigens, then cultured *in vitro* and returned to the patient to stimulate anti-cancer response. [Turner et al. J. Exp. Med. 190 (11), 1669-78 (1999) (Exhibit E)].

10. It is my considered scientific opinion that a PRO polypeptide shown to stimulate T-cell proliferation in the MLR assay of the present invention with an activity at least 180% of the control, as specified in the present application, is expected to have the type of activity as that exhibited by IL-12, and would therefore find practical utility as an immune stimulant. Some PRO polypeptides do the reverse, and give inhibition of T-cell proliferation in the MLR assay. It is my considered scientific opinion that a PRO polypeptide shown to inhibit T-cell proliferation in the MLR assay where the activity is observed as 80% or less of the control, as specified in the present application, would be expected to find practical utility when an inhibition of the immune response is desired, such as in autoimmune diseases.

Dated: 6/16/04

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Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Research Institute of Scripps Clinic,
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at
San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology, University of California at
Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State
University, San Francisco, California

Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological
Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco,
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9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical
Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research,
Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical
Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical
Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology
and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Research Grant, NIH (1985)

Recipient: P.I., Research Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the **Biochemical Subteam for Protropin® Project team.**

Served as Immunologist on the **Met-less hGH and Dnase project teams**, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in 1989-1990 on the **CD4-IgG project team** carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte $\beta 2$ integrins. Provided preclinical scientific data to **Anti-CD18 project team**

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule $\alpha 4\beta 1$ integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped establish and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against $\alpha 4\beta 7$ integrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reviewer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as Scientific advisor with staff of the **Business Development Office** on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The $\beta 2$ Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process -- Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process -- Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin $\alpha 4 \beta 7$ Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotypic Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent

Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent

5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and

Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and

Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams,

William Wood. U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William

Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood
US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

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2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. *J Immunol* 113:45-50, 1974.
3. Fong S: Studies on the relationship between the immune response and tumor growth. Ph D Thesis, 1975.
4. Benjamini E, Theilen G, Torten M, Fong S, Crow S, Henness AM: Tumor vaccines for immunotherapy of canine lymphosarcoma. *Ann NY Acad Sci* 277:305, 1976.
5. Benjamini E, Fong S, Erickson C, Leung CY, Rennick D, Scibienski RJ: Immunity to lymphoid tumors induced in syngeneic mice by immunization with mitomycin C treated cells. *J Immunol* 118:685-693, 1977.
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A number of agents can specifically or nonspecifically induce T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately proliferation of the activated T cells. Although proliferation is not a specific effector function of T lymphocytes—in contrast to helper function for B lymphocytes (UNIT 3.10) or cytotoxicity (UNIT 3.11)—proliferation assays are reliable, simple, and easy to perform and have been widely used to assess the overall immunocompetence of an animal. In addition, the assays described in this unit form the basis for identifying the appropriate cellular population that might be used to obtain T cell clones (UNIT 3.13) or T cell hybridomas (UNIT 3.14).

The assays have been divided into two groups on the basis of whether they are used to stimulate primed or unprimed T lymphocytes. The first basic protocol describes the use of agents that are capable of activating unprimed T lymphocytes in culture either by pharmacologic means (calcium ionophore and phorbol ester stimulation), by direct cross-linking of the T cell receptor (TCR) on a large percentage of responder cells (anti-CD3, anti-TCR- $\gamma\delta$, or anti-TCR- $\alpha\beta$ monoclonal antibodies), by cross-linking the receptors on certain subpopulations of T cells with monoclonal antibodies specific for the V regions of β chains of the TCR (anti-V β) or with enterotoxins specific for certain V β -chain regions, or by indirectly cross-linking the TCR (lectins or monoclonal antibodies to non-TCR antigens). The first alternate protocol describes the use of plate-bound antibodies specific for the TCR to stimulate proliferation. The second alternate protocol describes the activation of unprimed T cells to cell-associated antigens in the mixed leukocyte reaction (MLR). The first support protocol describes the preparation and use of T cell-depleted accessory or stimulator cells and the second support protocol describes methods for blocking accessory cell proliferation. Finally, the second basic protocol describes the induction of a T cell proliferative response to soluble protein antigens or to cell-associated antigens against which the animal has been primed *in vivo*.

The assays in this unit employ murine T lymphocytes. Induction of proliferative responses of murine B lymphocytes is described in UNIT 3.10. Related assays for use with human peripheral blood lymphocytes are described in UNIT 7.9.

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

ACTIVATION OF UNPRIMED T CELLS

Unprimed T cells can be induced to proliferate by a variety of agents, including pharmacological agents, anti-CD3/TCR or anti-Thy-1 monoclonal antibodies, enterotoxins and lectins. The commentary briefly describes the specificities of these agents, while Table 3.12.1 lists sources and concentrations for use in this protocol. Although this procedure is intended to measure proliferation of T cells specifically, in many cases induction of T cell proliferation is dependent on the presence of non-T cells that function as accessory cells. The latter provide additional costimulatory signals for T cell proliferation as well as cross-link (via their Fc receptors) monoclonal antibodies bound to cell-surface antigens. The requirement for non-T accessory cells varies with the nature of the stimulatory ligand and can range from absolute dependence to accessory cell-independent T cell activation (see Table 3.12.1). The activation is calculated after determining the difference in incorporation of [3 H]thymidine between stimulated and control cells.

BASIC PROTOCOL

In Vitro Assays
for Mouse B and
T Cell Function

3.12.1

Table 3.12.1 Agents Used to Activate Unprimed T Cells in Proliferative Assays

Agent ^a	Source/ cat. no. ^b	Concentration	Accessory cells ^c	Mode of action, etc.
PMA	SIG P8139	1-10 ng/ml	No	Use with ionomycin or A23187; pharmacologic
Ionomycin	CAL 407950	200-500 ng/ml	No	Use with PMA; pharmacologic
A23187	CAL 100105	100-500 ng/ml	No	Use with PMA; pharmacologic
PHA	WD HA16	1-5 µg/ml	Yes	Indirect TCR cross-linking
Con A	PH 17-0450-01	1-10 µg/ml	Yes	Indirect TCR cross-linking
Anti-Thy-1	PG mAb-G7	1-50 µg/ml	Yes ^c	Indirect TCR cross-linking
Anti-CD3	PG HM-CD3	0.1-5 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-αβ	PG HM-AB-TCR	0.1-10 µg/ml	Yes ^c	Use plate-bound or soluble; direct TCR cross-linking
Anti-TCR-γδ	PG HM-GD-TCR-1; HM-GD-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-8.1, 8.2 ^c	PG MM-Vβ-TCR-1	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-6 ^c	PG RM-Vβ-TCR-2	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Anti-Vβ-11	PG RM-Vβ-TCR-3	0.1-100 µg/ml	No	Use plate-bound; direct TCR cross-linking
Staph tox A	TT AT101	1-10 µg/ml	Yes ^c	Vβ-1,3,10,11,17-receptor specificity
Staph tox B	TT BT202; SIG S4881	1-100 µg/ml	Yes ^c	Vβ-3,7,8,17-receptor specificity
Staph tox E	TT ET404	1-10 µg/ml	Yes ^c	Vβ-11,15,17-receptor specificity

^aAbbreviations: PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; Con A, concanavalin A; Staph tox A, B, & E, *Staphylococcus enterotoxins* A, B, & E.

^bSupplier addresses and phone numbers are provided in APPENDIX 5. Abbreviations: CAL, Calbiochem; PG, Pharmingen; PH, Pharmacia LKB; SIG, Sigma; TT, Toxin Technology; WD, Wellcome Diagnostics.

^cWhen using anti-CD3 and anti-TCR antibodies in soluble form (rather than plate-bound), accessory cells are required. When using Staph enterotoxins, accessory cells must express appropriate MHC class II molecules. Accessory cell dependence is not absolute with anti-Thy-1 antibodies.

Materials

Complete RPMI-5 and RPMI-10 media (APPENDIX 2)

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (UNIT 3.1)

Activating agent(s) (Table 3.12.1)

Phosphate-buffered saline (PBS; APPENDIX 2)

Accessory cells: unfractionated mouse spleen cell suspension, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

[³H]thymidine (APPENDIX 3)

15- and 4-ml disposable, polystyrene conical tubes with screw caps

Low-speed centrifuge with Sorvall H-1000B rotor (or equivalent)

1-, 5-, and 10-ml disposable polystyrene pipets

96-well flat- or round-bottom microtiter plates with lids (Costar #3596 or #3799)

25- to 100- μ l single- and multichannel pipettors with disposable tips

Additional reagents and equipment for removing organs (UNIT 1.9), preparing single-cell suspensions (UNIT 3.1), and counting, labeling, and harvesting cells (APPENDIX 3)

1. Prepare responder leukocyte suspensions from thymus, spleen, or lymph node in complete RPMI-5 as described in UNIT 3.1.

The size of the intended experiment dictates the number of organs to be collected. See annotation to step 3 for an indication of cell number required, and UNIT 3.1 for number of cells per organ. Spleen, thymus, and lymph node can be used as responder cells, while only spleen is a source of accessory cells. Purified T cells or subpopulations of T cells (i.e., CD4⁺ or CD8⁺) cells may also be used. See UNITS 3.1-3.6 for enrichment/depletion methods.

2. Centrifuge single-cell suspensions in 15-ml conical tubes for 10 min in Sorvall H-1000B rotor at ~ 1000 rpm ($200 \times g$), room temperature, and discard supernatant.
3. Resuspend cell pellet in complete RPMI-5. Count responder cells and adjust to $\sim 10^6$ cells/ml with complete RPMI-10.

While this concentration (1×10^6 cells/ml or 2×10^5 cells/well) will give satisfactory responses with most cell populations, it is useful to compare 2, 4, and 8×10^5 cells per well in initial pilot experiments. If unfractionated spleen or lymph node cells are used as the responder population, sufficient accessory cells are present and there is no need to supplement the cultures with additional cells. However, if highly purified T cells or T cell subpopulations are used as responders, it will be necessary to add non-T accessory cells depending on the nature of the activating agent (see Table 3.12.1). This is most easily accomplished by adding increasing numbers (0.1 , 0.5 , and 1.0×10^5) of syngeneic spleen (accessory) cells in 0.1 ml to 2×10^5 T cells in 0.1 ml (see first support protocol). Also, a meaningful comparison of the responsiveness of different cell populations requires titrations of both the activating agents as well as the responding cell populations, and a kinetic experiment.

4. Prepare working solutions of activating agents in 4-ml conical tubes at room temperature as follows. For MAb, toxin, or lectin, make a series of four dilutions from 1 mg/ml stock solutions—e.g., 100, 30, 10, and 3 μ g/ml in PBS. For the pharmacological agent, make single dilutions of 100 ng/ml solution of PMA and 1 μ g/ml A23187 (or 4 μ g/ml ionomycin) in PBS.

If MAb in supernatant or ascites form are being used, at least four dilutions should also be used. Working solutions should be used immediately, since the various proteins, especially MAb, may bind to the plastic.

In Vitro Assays
for Mouse B and
T Cell Function

3.12.3

See Table 3.12.1 for V β specificities of staphylococcal enterotoxins. It is essential to verify that the mouse strain employed expresses the MHC class II surface molecules for which the enterotoxin has a specific binding affinity. See Marrack and Kappler (1989) for further discussion of various enterotoxins and their specificities.

5. Add 20 μ l of each dilution of activating reagent (MAb, enterotoxin or lectin) to each of three wells of a 96-well flat- or round-bottom microtiter plate. Include control wells with 20 μ l of PBS only. Add 20 μ l PMA or calcium ionophore at the single concentration indicated in step 4, as the dose-response curve for these agents is extremely narrow.

A series of four dilutions will form one row of each microtiter plate, allowing for efficient organization of the plates.

6. To the wells of the 96-well microtiter plate containing activating agent, add 2×10^5 cells in 0.2 ml.
7. Place microtiter plates in a humidified 37°C, 5% CO₂ incubator for 2 to 4 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions and must be determined empirically (see critical parameters).

8. Add [³H]thymidine to each well. Return the plates to CO₂ incubator to pulse 18 to 24 hr. Harvest cells using a semiautomated sample harvester and measure cpm in β scintillation counter.
- 9a. Compute the data as the difference in cpm of stimulated (experimental) and control (no activating agent added) cultures. This is done by subtracting the arithmetic mean of cpm from triplicate control cultures from the arithmetic mean of cpm from corresponding stimulated cultures. The results are referred to as " Δ cpm."
- 9b. Alternatively, compute the data as the ratio of cpm of stimulated and control cultures. This is done by dividing the arithmetic mean of cpm from stimulated cultures by the arithmetic mean of cpm from control cultures. The results are referred to "SI" (stimulation index).

The second method (step 9b) has the disadvantage that small changes in background values will result in large changes in SI and should be interpreted with caution. In most publications, Δ cpm rather than SI values are preferred.

ALTERNATE PROTOCOL

ACTIVATION OF UNPRIMED T CELLS WITH PLATE-BOUND ANTIBODIES

Although it is possible to induce T cell activation with monoclonal antibodies to the CD3/TCR complex in solution during culture, such activation depends on cross-linking of the antibody by Fc receptor-bearing accessory cells. This protocol describes the use of monoclonal antibodies to the CD3/TCR complex by coupling them to the wells of the microtiter plates. The T cell proliferative response induced under these conditions does not require the presence of significant numbers of accessory cells, although the responses obtained may be suboptimal (Jenkins et al., 1990).

Use of this protocol is recommended for use with those antibodies to the CD3/TCR complex which bind poorly to the Fc receptor present on murine accessory cells and which do not induce T cell activation in soluble form. Although all monoclonal antibodies readily couple to plastic under these conditions, it is very difficult to induce a proliferative response with certain antibodies such as the G7, anti-Thy-1 monoclonal antibody. In such cases, the conditions described in the basic protocol should be followed.

Additional Materials

PBS (APPENDIX 2), room temperature and 4°C

1 mg/ml purified anti-CD3 or anti-TCR MAb in PBS (for nonspecific activation of T cells) or 1 mg/ml purified anti-V β or anti-TCR- $\gamma\delta$ MAb in PBS (for activation of T cells with specific receptors; see Table 3.12.1)

1. In 4-ml conical polystyrene tubes, prepare a series of four dilutions of MAb from sterile 1 mg/ml stock solutions—e.g., 100, 10, 1, and 0.1 μ g/ml—using room temperature PBS.

Sources and recommended concentrations of monoclonal antibodies can be found in Table 3.12.1; since MAb will bind to plastic, the working dilutions should be used immediately.

The ability of anti-TCR antibodies to cross-link receptor molecules varies depending on the purity of the MAb preparation and the affinity of the MAb for the TCR/CD3 complex. Optimum dilutions will have to be determined in dose-response experiments. Alternatively, preparations of ascites fluid from the MAb can be tested at different dilutions (e.g., 1:100, 1:200, 1:400, and 1:800), but use of purified antibody will allow for better standardization of the assay.

Because the efficacy of MAb-induced activation depends on the amount of antibody bound to the bottom of the wells, it is crucial to make the dilutions in a buffer without any additional source of proteins such as FCS or albumin; these would compete with the binding of the antibody, and therefore reduce the responsiveness. For this reason, it is also not recommended to perform the assay with culture supernatants of the appropriate hybridomas.

2. Add 30 μ l of each concentration of MAb solution to each of three wells of a 96-well round-bottom microtiter plate. Include control wells of 30 μ l PBS only.

A series of four dilutions will form one row of each plate, allowing for efficient organization of the plates. Consistently better responses are seen with round-bottom (compared with flat-bottom) plates in antibody-mediated experiments.

Most often, optimal responses are seen with 10 μ g/ml antibody. There is no point in adding more than the indicated amount of antibody, since the maximum amount that can bind to surface of the wells is \sim 2 to 3 μ g (A.M.K., unpub. observ.).

3. Cover the plate and gently tap its side to ensure complete covering of the bottom of the wells. Incubate plates 90 min at 37°C. During incubation, proceed to step 4.

During this incubation, the antibodies bind to the plastic in the wells for subsequent cross-linking of the T cell receptors on responding T cells. Plates can also be prepared the night before an experiment and kept in the refrigerator overnight, after the 37°C incubation.

4. Prepare responder cell suspensions as in steps 1 to 3 of the basic protocol.

Highly purified T cell populations can be used in these studies as the proliferative response induced is accessory cell-independent. However, the presence of non-T accessory cells does not interfere with the proliferative response.

5. Wash the wells of the incubated plates by adding 200 μ l cold PBS and inverting the plates with a flick of the hand on a stack of paper towels placed in a tissue culture hood. Repeat washing procedure two more times to remove excess antibody.

6. To the wells of the washed plates, add $\sim 2 \times 10^5$ cells in 0.2 ml.

If cells are not ready at this stage, plates may be kept in the refrigerator overnight after 100 μ l PBS has been added. Presumably, longer storage periods should be acceptable, but our experience is limited to ≤ 4 day periods. The PBS should be removed before the cells are added.

Most cell populations will give peak responsiveness at this cell dosage, but pilot experiments should be performed to establish optimal conditions.

7. Proceed as in steps 7 to 9 of the basic protocol, but incubate cultures for 2 to 3 days before adding [^3H]thymidine.

Kinetic assays should be performed to determine the optimum culture period.

ALTERNATE PROTOCOL

T CELL PROLIFERATION IN MIXED LYMPHOCYTE CULTURES

In the mixed lymphocyte culture (MLC) or reaction (MLR), suspensions of responder T cells are cultured with allogeneic stimulator lymphocytes. The activating stimulus is the foreign histocompatibility antigen (usually MHC class I or class II molecules) expressed on the allogeneic stimulator cells. Responder cells need not be primed because a sufficiently high number of T cells in the MLC will respond to the stimulator population. If the stimulator cell population contains T cells, their uptake of [^3H]thymidine must be prevented by irradiation or treatment with mitomycin C; alternatively the stimulator cell suspension can be depleted of T cells (see support protocols).

Additional Materials

Responder cells: lymphocytes from nonimmunized mouse thymus, spleen, or lymph nodes (UNITS 1.9 & 3.1) or purified T cells or T cell subpopulations (UNITS 3.1-3.6)

Stimulator cells: allogeneic mouse spleen cells that differ from the responder cells at H-2 or MIs loci, irradiated or treated with mitomycin C (second support protocol) or T cell-depleted (first support protocol)

1. Prepare responder cell populations as in steps 1 to 3 of the basic protocol. Although unfractionated cell populations can be used as responders in certain situations, it may be preferable to use purified T cells or T cell subsets.

To estimate the MLR of a cell population, it is necessary to perform a dose-response assay with different numbers of responder cells. Typically, three replicate wells are set up containing each of the following: 0.5×10^5 , 1×10^5 , 2×10^5 , and 4×10^5 cells (optimal responses are usually obtained with the latter two densities). The setup for these four cell densities will occupy one row (12 wells) of a microtiter plate.

For thymocytes, it may be necessary to use 8×10^5 cells per well because the frequency of responding T cells is lower; the lowest number of responder cells could then be 1×10^5 and the doses in between would be 2×10^5 and 4×10^5 . Using this range of higher numbers of responder cells may also be preferred when experimental manipulations are expected to reduce the frequency of responding T cells.

2. To a 96-well microtiter plate, add 5×10^4 to 4×10^5 responder cells in 0.1 ml to each well. For each experimental group, set up three replicate wells.

Stimulation of leukocytes for proliferation in 96-well microtiter plates can be run in parallel with cytotoxic T lymphocyte (CTL) generation (UNIT 3.11), which is performed in 24-well microtiter plates. For example, cells can be diluted to 4×10^6 cells/ml and added to 24-well plates in 1.0 ml/well for CTL generation and to 96-well plates in 0.1 ml/well for proliferation.

3. Prepare a single-cell suspension of irradiated or mitomycin C-treated stimulator cells. Alternatively, prepare a suspension of T-cell depleted stimulator cells. Add 0.1 ml to each well of the plates containing responder cells.

The optimum number of stimulator cells must be determined for each MLC and for different responder cells. For a range of responder cells from 0.5 – 4×10^5 , test stimulator cells at densities of 2, 4, and 8×10^5 /ml (i.e., 2, 4, and 8×10^5 /well). It should be noted that the stimulator cell suspension provides both the specific antigen to be recognized by the responder T cells as well as nonspecific accessory cells. If

highly purified T cells are used as the responder population, it is therefore not necessary to supplement the cultures with non-T accessory cells syngeneic to the responder T cells.

Separate wells with control cultures should be set up that include—for each dose of responder and stimulator cells—replicate wells of responder cells with irradiated or mitomycin C-treated syngeneic stimulator cells. Values obtained from these controls reflect “background” proliferation values (see step 9 of basic protocol). Other negative controls often included are wells with stimulator cells alone and wells with responder cells alone. These are not used for the calculation of the data, but are useful to compare with the background proliferation values; the latter should not be much higher (<2-fold) than those obtained with stimulator or responder cells alone. Higher background values indicate potential autoreactivity.

4. Follow steps 7 to 9 of the basic protocol, but incubate the cultures for 3 to 6 days.

Optimum culture periods for stimulating cells will vary depending on cell type and laboratory conditions, and must be determined empirically (see critical parameters).

DEPLETION OF T CELLS FROM ANTIGEN-PRESENTING/STIMULATOR CELL SUSPENSIONS

Although normal unfractionated spleen cell populations can be used as a source of accessory cells, in certain types of experiments it may be preferable to use spleen cell populations from which the T cells have been removed. This procedure ensures that none of the observed proliferative responses of the responder population result from T cell factors derived from the accessory cell population. For example, even T cells whose cell division has been blocked (second support protocol) can produce cytokines. In the following steps, T cell-depleted spleen cell suspensions are prepared using a lytic monoclonal antibody to the T cell antigen, Thy-1. Because almost all the antigen presentation or stimulator cell activity in spleen resides in the non-T cell fraction, this procedure also leads to enrichment of functional antigen-presenting cell function. Further enrichment of antigen-presenting cells (APC) by flotation of the T cell-depleted spleen cells on Percoll gradients is also described. Other procedures leading to enrichment of APC are described elsewhere; the method described in UNIT 3.7 does not deplete T cells and therefore is not recommended here; the method described in UNIT 3.15 leads to higher levels of enrichment that are not required in the protocols presented here.

Additional Materials

- Spleen cells from nonimmunized mice
- Hanks balanced salt solution (HBSS; APPENDIX 2)
- Low-Tox rabbit complement (Cedarlane #CL3051), reconstituted with ice-cold distilled water and filter-sterilized
- Anti-Thy-1.2 ascites (HO-13-4; ATCC #TIB 99) or anti-Thy-1.1 ascites (HO-22-1; ATCC #TIB 100; alternatively, see Table 3.4.1 for other anti-Thy-1 MAb and UNIT 2.6 for production of ascites)
- 70% Percoll solution (UNIT 3.8 and reagents and solutions)

1. Centrifuge the spleen cell suspension derived from single spleen down to a pellet.

The spleen cells should always be from nonprimed animals and should be syngeneic to the responder T cells unless they are to be used as stimulator cells in the MLC.

2. To the pellet, add 0.9 ml HBSS, 0.1 ml complement, and 25 μ l anti-Thy-1 ascites.

If cells from more than a single spleen are needed, the procedure should be scaled up accordingly.

SUPPORT PROTOCOL

In Vitro Assays
for Mouse B and
T Cell Function

3.12.7

The choice of anti-Thy-1 reagent to be used depends on the strain of animal from which the spleen was derived. The great majority of commonly available mouse strains (except AKR) express the Thy-1.2 allele.

3. Incubate the mixture at 45 min in a 37°C water bath.
4. Centrifuge 10 min in Sorvall H-1000B rotor at ~1000 rpm (200 × g), room temperature, and discard supernatant. Resuspend pellet in HBSS and wash two more times.
5. Count viable cells (APPENDIX 3) and resuspend in complete RPMI-10 or PBS for inactivation as in the second support protocol, or in HBSS to prepare low-density accessory cells (see below).

The T cell-depleted spleen cell population is comprised of B cells, macrophages, and dendritic cells. Further enrichment of cells with enhanced accessory cell function can be obtained by fractionation of this population on Percoll.

6. Dilute 70% Percoll solution to 55% by mixing 23.58 ml of the 70% Percoll with 6.42 ml HBSS. Resuspend T cell-depleted spleen cells from step 5 in HBSS at 20×10^6 cells/ml.
7. Layer 3 ml cell suspension over 3 ml of 55% Percoll solution in a 15-ml conical centrifuge tube.
8. Spin 13 min in H-1000B rotor at 3000 rpm (1900 × g), room temperature.
9. Remove cells that band at the Percoll/HBSS interface with a 5-in. Pasteur pipet and wash 3 times in HBSS as in step 4.
10. Count viable cells and resuspend in complete RPMI-10 for inactivation according to the second support protocol.

The population obtained from steps 6 to 10 is comprised of large cells including macrophages, dendritic cells, and activated B lymphocytes. This population of cells is enriched in accessory cell function. When used in either of the basic protocols with purified T responder cells, fewer of the Percoll-purified cells should be needed to provide accessory function.

SUPPORT PROTOCOL

BLOCKING CELLULAR DIVISION OF ACCESSORY/STIMULATOR CELLS

There are two situations in which inhibition of accessory or stimulator cell division should be blocked. When purified T cells rather than unfractionated lymphoid populations are used in the basic protocol, cultures are frequently supplemented with accessory cells syngeneic to the responder T cells. If accessory cell DNA synthesis is inhibited, one can then be certain that the resultant proliferative response is comprised entirely of responder T cells and does not contain a component of recruited B cell proliferation derived from the accessory cell populations. In the MLR, the stimulator cells are spleen cells from mice that differ from the responder cells in *H-2* and/or *Mls* gene expression (see APPENDIX 1, Tables A.1C.1 and A.1F.1) and they can also recognize alloantigens on the responder cells. This responsiveness of stimulator cells against responder cells in an MLR (so-called back-stimulation) must be prevented by blocking cellular division. This can be done by treatment of stimulator cells with mitomycin C (a DNA cross-linking reagent) or by γ irradiation. Many investigators prefer mitomycin C treatment when antigenic differences encoded for by *Mls* genes are to be measured, or when an irradiation source is not available. For more information on the loci encoding *Mls* genes, see Tables A.1F.2 and A.1F.3.

Mitomycin C Treatment

Additional Materials

Mitomycin C (Sigma #M-0503; store in dark)

1. In a 15-ml aluminum foil-wrapped tube, prepare a solution of mitomycin C in PBS at 0.5 mg/ml and filter sterilize.

Since mitomycin C is very light-sensitive, it is necessary to prepare a fresh stock solution each day for each experiment.

2. Prepare spleen cell suspension as described in steps 1 and 2 of the basic protocol at a concentration of 5×10^7 cells/ml in PBS.
3. Add mitomycin C to a final concentration of 50 μ g/ml (100 μ l/ml of cell suspension) and wrap the tube in aluminum foil. Incubate 20 min at 37°C.
4. Add an excess of complete RPMI-5 (i.e., fill tube with ~12 ml) and centrifuge 10 min in Sorvall H-1000B rotor at 1200 rpm ($300 \times g$). Discard supernatant and repeat washing procedure two more times.

Three washes are crucial, because any traces of mitomycin C left among the cells will reduce proliferative responses when the cells are added to an MLC.

5. Resuspend pellet in complete RPMI-10. Count cells with hemacytometer. Adjust to desired concentration as described in the annotation to step 6 of the basic protocol.

Irradiation Treatment

Prepare a spleen cell suspension as described in steps 1 to 3 of the basic protocol, at a final concentration of $5\text{--}10 \times 10^6$ cells/ml in complete RPMI-10. Using a source of ionizing irradiation (^{60}Co or ^{137}Cs γ -irradiator; e.g., Gammacell 1000, Nordion), deliver 1000 to 2000 rad of irradiation to the cells.

This dose range of irradiation is suitable for most immunologic applications employing spleen cell suspensions. However, antigen presentation by different spleen cells is differentially affected by irradiation (Ashwell et al., 1984): at low doses (500 to 1000 rad), antigen-presenting function of B cells is preserved; after doses of 1100 to 2000 rad, a substantial decline is observed; and doses >2000 rad abolish the participation of B cells as APC. Macrophages and dendritic cells, on the other hand, maintain antigen presentation through doses of 3000 rad. To ensure that B cells do not participate in the responses measured, some investigators prefer to use doses of 2000 rad. However, responsiveness to *Mls* antigens can best be measured with stimulator cells that received doses of <1000 rad, since B cells present *Mls* more effectively. Alternatively, *Mls* responsiveness can be measured after mitomycin C treatment of stimulator cells, since it also preserves the antigen-presentation function of B cells.

When transformed cell lines are used as antigen-presenting or accessory cells, higher doses must be used to ensure blockage of cell division. The appropriate dose will have to be determined empirically for each cell line, but is likely to be at least 5000 rad; some transformed cell lines require as much as 10,000 to 12,000 rad, and may be more sensitive to mitomycin C treatment.

ACTIVATION OF PRIMED T CELLS

Proliferative responses to viruses, protein antigens, minor transplantation antigens, and the male H-Y antigen require in vivo immunization followed by in vitro stimulation. Furthermore, enhanced proliferative responses to those antigens that will generate primary in vitro responses (i.e., MHC antigens) can be obtained by in vivo priming. Multiple immunizations usually elevate in vitro responses.

To immunize animals for in vitro secondary responses to soluble protein antigens or peptides, dissolve antigens and emulsify in complete Freund's adjuvant (UNIT 2.5). For strong responses by draining lymph node cells, immunize animals in a hind footpad. For

BASIC PROTOCOL

In Vitro Assays
for Mouse B and
T Cell Function

3.12.9

strong responses by spleen cells, immunize intraperitoneally. Tail-base immunization also can be used as an efficient route of immunization; follow procedure for intradermal injection. To prime animals against cellular antigens, inject intraperitoneally with $1-5 \times 10^7$ cells that express the antigen. Immunization protocols are described in UNIT 1.6.

Within 2 to 3 weeks after in vivo priming, in vitro responsiveness of primed T cells can usually be measured. This assay is often used as a preparation for subsequent in vitro cloning procedures (UNIT 3.14) and T cell hybridoma preparation (UNIT 3.13).

Materials

Complete RPMI-10 medium (APPENDIX 2)

Responder cells: Purified T cells isolated from lymph nodes (UNITS 3.1-3.6) of in vivo primed mice

Antigen: 1 mg/ml sterile protein antigen(s) (UNIT 3.13), in PBS or suspension of irradiated or mitomycin C-treated stimulator cells expressing alloantigens at 8×10^6 cells/ml (UNIT 3.11, support protocol) in complete RPMI-10 medium (APPENDIX 2)

Accessory cells: suspension of irradiated or mitomycin C-treated (or T cell-depleted) spleen cells syngeneic to the responding T cells at 5×10^6 cells/ml in complete RPMI-10 medium

4-ml conical tubes

96-well flat-bottom microtiter plates with lids

1. Follow steps 1 to 3 of the first basic protocol for preparation of responder cells.
2. Prepare 4-fold dilution series of the antigens in 4-ml conical tubes, using complete RPMI-10.

The following dilutions are recommended: 100, 10, 1, and 0.1 μ g/ml protein antigens and 8, 4, 2, and 1×10^6 cells/ml of stimulator cells in complete medium.

3. Add antigens to 96-well flat-bottom microtiter plates, at 30 μ l/well for protein antigens or 100 μ l/well for cellular antigens. For each experimental group, set up three replicate wells and include control wells with medium only (no antigen).

By using four concentrations of antigens and three replicate wells for each dose, one row of a microtiter plate will cover the entire tested range.

4. Add responder T cells in 0.1 ml to each well.

Purified T cells are recommended; otherwise extremely high background values may be obtained. This appears to be due in part to proliferation of recruited cells (T and non-T) that are not antigen-specific. If unfractionated lymph node cells from recently primed mice are used, add $1-2 \times 10^5$ cells per well and proceed to step 6.

5. If purified lymph node T cells specific for protein antigens are used, add 0.1 ml of accessory spleen cells syngeneic to the donor of the responder T cells at 5×10^5 cells per well.

Purified T cells require an exogenous source of accessory non-T cells. Accessory cells function both as antigen-presenting cells and as a source of undefined "second signals." They are not required for cell preparations primed against cellular antigens, because accessory cell function is provided by the stimulator cells.

6. Proceed as in steps 7 to 9 of the basic protocol.

Culture periods before labeling can vary widely and kinetic assays should be performed. In general, for T cells from primed mice, it is likely that the response will peak at day 4 or 5.

REAGENTS AND SOLUTIONS

Percoll solution

Diluent:

45 ml 10× PBS, pH 7.4 (APPENDIX 2)

3 ml 0.6 M HCl

132 ml H₂O

Filter sterilize

70% Percoll solution:

63 ml Percoll (Pharmacia LKB #170891-01)

37 ml sterile diluent (above)

Final osmolarity should be 310 to 320 osM

COMMENTARY

Background Information

Proliferative assays for measuring T cell function have certain advantages and disadvantages compared to the cytotoxic T lymphocyte (CTL) assay described in UNIT 3.11 or the lymphokine production assays in UNITS 3.15 & 6.3. Advantages are that proliferative assays are less time-consuming, less labor-intensive, less cell-consuming, and less expensive than "true" effector T cell function assays. A disadvantage is that antigen specificity is not as easily demonstrated in proliferative assays as in CTL assays, unless antigen-specific clones of proliferating cells are used. Furthermore, the proliferative assay only detects dividing cells instead of measuring true effector T cell function.

It is not clear which T cell function is measured in proliferative assays; the proliferative response should therefore be used solely as general indicators of T cell reactivity. Data obtained in proliferative assays might variously reflect proliferation of CTL, lymphokine-producing T cells, or nonactivated "bystander" cells, and will be severely affected by the function of non-T cells such as accessory cells (see below). Since the majority of T cells respond to and produce IL-2 upon activation, differences in responsiveness in a proliferative assay in part reflect differences in IL-2 production by the responding T cells. Proliferative assays therefore become more meaningful when combined with the lymphokine detection assays presented in UNITS 3.15 & 6.3. Since responsiveness to IL-2 is also determined by the levels and functionality of IL-2 receptors, further information will be added by including measurements of IL-2 receptors (UNIT 6.1) or by flow cytometry (UNIT 5.4). Yet, as a first approximation of cellular activation, proliferative assays are valuable.

Critical Parameters and Troubleshooting

Parameters affecting the magnitude of T cell proliferative responses include cell concentration, type of medium, source of serum, incubator conditions (CO₂ level and humidity), type and concentration of activating agent, type of responding T cells, type of accessory/stimulator cells, mouse strain, and culture time. Optimal conditions for individual laboratories and experiments must be derived empirically with respect to these variables, but general guidelines are provided below.

A number of agents can be employed in the first basic protocol to induce T cell proliferation (Table 3.12.1). T cells may be activated by pharmacologic means by producing an elevation of intracellular free calcium with a calcium ionophore combined with activation of protein kinase C with a phorbol ester. The most direct means of inducing T cell activation involves stimulation with monoclonal antibodies that interact with the CD3/TCR complex—i.e., anti-CD3, anti-TCR- $\alpha\beta$ or - $\gamma\delta$, as well as anti-V β antibodies that are capable of interacting with a subset of cells bearing a specific TCR. A vigorous T cell proliferative response of defined subsets can also be induced with certain bacterial toxins known as staphylococcal enterotoxins. These toxins are often referred to as "superantigens" (Marrack and Kappler, 1989) because they stimulate T cells via the variable (V) gene segment of the TCR. Different toxins have affinities for different V β chains and these specificities make them valuable reagents for activating T cells. The activating capacity of toxins is also dependent on their ability to bind to MHC class II molecules (i.e., responding T cells react with the toxin/class II complex); thus, responsiveness varies with the

**In Vitro Assays
for Mouse B and
T Cell Function**

3.12.11

mouse strain used. Lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) have been widely used for many years to activate T cells. Although the precise mechanism of action of these agents is unknown, it is likely that lectins activate T cells by indirectly cross-linking the TCR because TCR-negative cells will not respond to these agents. Lastly, it is also possible to induce T cell activation with monoclonal antibodies to cell-surface antigens other than the TCR; this protocol employs the G7 monoclonal antibody, one of the most effective of the anti-Thy-1 activators (Gunter et al., 1984).

When comparing the reactivity of different cell populations, it is essential to perform dose-response assays for responder T cells and activating agents and for both responder and stimulator T cells (in MLR), since each population may yield optimal responses at different cell numbers. This may reflect differences in frequency of responding cells, and hence may indicate a need to perform limiting dilution assays (UNIT 3.15). Since peak responsiveness of different populations of T cells may occur at different times, it is also essential to perform kinetic experiments—i.e., compare responsiveness at days 2, 3, 4, and 5.

Differences in responsiveness need not necessarily be due to differences in the frequency of responding T cells, but may also indicate differences in the efficacy with which co-stimulatory activity or “second signals” are delivered by the accessory cells present in different cell populations. The type of interactions pertinent to the generation of primary responses by T cells is explained in the commentaries of UNITS 3.8, 3.11, & 3.13. Specific requirements for inducing activation with immobilized antibodies have been described (Staerz and Bevan, 1986; Hathcock et al., 1989; Jenkins et al., 1990). A responding cell population completely devoid of accessory cells (such as purified populations of splenic or lymph node T cells or cloned T cells) will yield fine responsiveness in an MLC, since accessory cell function is provided by the stimulator cells; however, the same population will generally not yield responses when mitogens, antigens, or enterotoxins are used. In such a setting, accessory cells may also function as antigen-presenting cells (APC). Addition of irradiated or mitomycin C-treated syngeneic sources of accessory cells (either whole spleen cells or purified APC; see first support protocol) can be used to restore responsiveness in purified T cells. The need for accessory cells can sometimes be

bypassed when anti-TCR monoclonal antibodies are coupled to plastic, or when certain anti-Thy-1 monoclonal antibodies are used; however, these conditions do not necessarily result in optimal responsiveness (Jenkins et al., 1990).

The level of [3 H]thymidine incorporation should not be regarded only as a reflection of cellular proliferation: some nondividing cells will synthesize DNA and “cold” thymidine released by disintegrating cells will compete with incorporation of labeled thymidine. Therefore, measurements of DNA synthesis should be accompanied by counting viable cells over the length of the culture period if a true estimate of cellular proliferation is to be obtained. Of course, cell death of nonactivated cells will also interfere with the accuracy of this last parameter.

The sensitivity of proliferation assays is such that small errors in cell numbers will result in large differences in [3 H]thymidine incorporation values. When values obtained in triplicate cultures correspond poorly (e.g., >5% difference in cpm values >1000), technical problems such as cell clumping, dilution, and pipetting should be considered. Excessively high values may be obtained from contaminated wells, as [3 H]thymidine will be incorporated into replicating bacteria; therefore, it is good practice to check the wells from microtiter plates under an inverted microscope for contamination. Contamination may also interfere with proliferation of the activated lymphocytes.

It is also useful to check for blast formation by microscopic examination of the cultures: activated lymphocytes will tend to enlarge, and detection of blasts will give a general indication of successful activation.

The main problem that may occur with proliferative response assays is high levels of background [3 H]thymidine incorporation in control cultures without antigens. This problem is frequently due to the fetal calf serum (FCS) used to supplement the cultures, which may be mitogenic for B cells. Different lots of FCS should be screened to select those that are nonstimulatory or only weakly stimulatory in the absence of other stimuli, and that support strong proliferative responses upon antigenic stimulation of T cells.

If flat-bottom microtiter plates are used in the procedure and weak responses occur, it may be useful to switch to round-bottom plates. Our laboratory has found consistently better responses in round-bottom plates when

thymocytes are used as responder cells or with slight alloantigenic differences between responding and stimulating cells. In addition, antibody-mediated experiments yield better results with round-bottom plates. Presumably, this reflects better cell contact obtained in such plates; optimal responses will almost certainly occur at different cell numbers than in flat-bottom plates and densities will have to be adjusted accordingly.

Although satisfactory responses to most alloantigens can be obtained with complete RPMI-10 medium, it may be necessary to compare different media. This need arises when the proliferative responses are weak (i.e., when [^3H]thymidine values for activated cultures are <10-fold higher than those for control cultures) and may occur under various circumstances: weak alloantigenic differences between responder and stimulator cells, weak T cell proliferative function in the responder cells or diminished APC function in the stimulator cells due to experimental manipulations, or a low precursor frequency of responding T cells. Thymocytes in particular do not contain a high level of responding T cells. Frequently, proliferation can be improved when complete Clicks or Dulbeccos media are used (with additives as described in APPENDIX 2), presumably because these media contain additional nutrients and have an osmolarity more compatible with mouse serum than RPMI.

When RPMI is used as medium, 5% CO_2 will be sufficient, but for other media, a 7.5% CO_2 concentration in the incubator will be more satisfactory. Generally, the buffering capacity of DMEM is insufficient at 5%, but fine at 7.5%. Much will also depend on the proliferative activity of the responding population of T cells (e.g., vigorous proliferation will reduce the pH in the cultures); it is therefore recommended to compare responsiveness in initial pilot experiments in incubators set at different CO_2 concentrations.

The culture period required for stimulation—after which the cells are to be labeled—varies for different laboratories, media, and types of responding and stimulator cells. Conditions eliciting weak responses, such as those obtained with thymocytes or a weak alloantigenic difference, will require a longer culture period (5 to 6 days) than those which elicit a higher frequency of responding T cells (3 to 4 days). Because laboratory conditions vary, it will be necessary to run a kinetic assay to determine the optimal time for T cell prolifer-

ation. Addition of [^3H] thymidine on days 2, 3, 4, 5, and 6 will provide a useful test; further extension of the culture period will not yield any improvements, due to exhaustion of nutrients in the medium.

Anticipated Results

For proliferative assays described in the basic protocol, which activate the majority of the responding T cells, responses of 100,000 cpm should be obtained; in the MLR or following activation with monoclonal antibodies to subpopulations of T cells (anti-V β), responses up to 100,000 cpm may be observed; however, measurements of 20,000 cpm (with tight standard errors) can be quite satisfactory. Background values of <1000 cpm should be expected. Reported results (as described in step 9a) should be mean cpm of experimental wells minus background cpm (Δ cpm).

Time Considerations

The time required to set up proliferative assays is not more than a day, with the number of hours depending on the number of different groups of responder cells that must be prepared. The time required for incubation of cells ranges from 2 to 6 days, as noted above in critical parameters. Following an additional 18- to 24-hr incubation period for pulsing, harvesting the cells and measuring cpm will require several hours depending on the number of plates (~15 min for harvesting each plate and ~100 min for counting each plate at 1 min/sample).

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Details the MLC proliferation assay.

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Dendritic cells are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

The Dendritic Cell Advantage: New Focus For Immune-Based Therapies

by Ralph M. Steinman

The focus of immune therapeutics has been on lymphocytes, the cellular mediators of immunity, and the suppression of lymphocyte function. The drug ciclosporin (cyclosporine) is an excellent and successful example. However, medicine needs therapies that enhance immunity or resistance to infections and tumors. Medicine also needs strategies, whether suppressive or enhancing, that are specific to the disease-causing stimulus or antigen. In contrast to lymphocytes, dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. DCs capture antigens and then initiate and control the activities of lymphocytes, including the development of resistance to infections and tumors (reviewed in references 1-3).

Summary

Dendritic cells (DCs) provide a much earlier and antigen-specific means for manipulating the immune response. The best-studied function of DCs is to convert antigens into immunogens for T cells. The "DC advantage" entails a myriad of functions. DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity. Another special feature of DCs is their location and movement in the body; DCs are stationed at surfaces where antigens gain access to the body. The events that make up the life history of DCs are now being unraveled in molecular terms. As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent. © 2000 Prous Science. All rights reserved.

The controlling role of DCs is best known for thymus-dependent lymphocytes or T cells which are important in many diseases, the most poignant being the AIDS epidemic (Table I). DCs were identified in a few laboratories that were focusing on the induction of immunity from resting T cells. It was noted that immune tissues (spleen, lymph nodes, lymph. blood) had a small fraction of cells with unusual

"tree-like" or "dendritic" processes. These distinctive cells had not been recognized previously and they proved to have distinct functions. Most importantly, DCs were potent inducers of immunity even in animals, not just the test tube, and now even in patients (reviewed in references 1-3).

The DC field was held back by the fact that there were so few cells relative

TABLE I: HUMAN DISEASES THAT INVOLVE T CELLS

- Rejection of organ transplants and graft-vs.-host disease in bone marrow transplantation
- Resistance to many infections including vaccine design
- Vaccines against tumors and immune therapies for existing tumors
- Allergy
- AIDS
- Autoimmune diseases like insulin-dependent (juvenile) diabetes, multiple sclerosis, rheumatoid arthritis and psoriasis

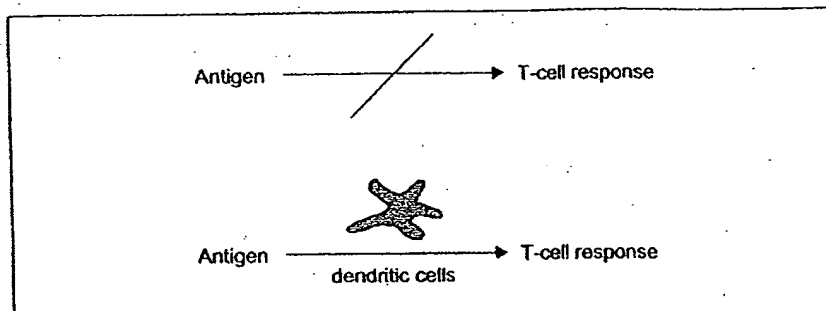


Fig. 1. A key function of dendritic cells. Antigens within tumors, transplants and infectious agents need to be presented by DCs to become immunogens, i.e., to make T cells begin to grow and exhibit their helper and killer functions.

to other players in the immune system such as B cells, T cells and macrophages. In reality, DCs are quite abundant for the job they have to do, namely, to initiate immune responses from antigen-specific T cells. In immune system organs like lymph nodes, DCs form an extensive network throughout the T cell-rich regions and physically outnumber any given antigen-reactive T cell by at least 100 to 1. The DC field was also held back because many thought that the cells were no different from macrophages, thus keeping investigators from working on the active DCs. In reality, DCs were identified on the basis of profound differences from macrophages, and their many distinct properties and functions were only uncovered by separating DCs from macrophages.

The best-studied function of DCs is to convert antigens into immunogens for T cells. The antigen receptors on T cells do not focus on intact proteins in microbes and tumors, but instead recognize fragmented or processed proteins, that is, peptides. The processing of protein antigens into peptides occurs within cells, and then the peptides are

displayed or presented at the cell surface affixed to products of the major histocompatibility complex (MHC). The ensuing interaction between a T-cell receptor (TCR) and its specific MHC-peptide complex allows a T cell to detect peptides formed within cells in transplants, tumors, sites of infection and self tissues attacked during autoimmune disease (Table I). "Antigens" refers to specific substances recognized by the immune system, while "immunogens" refers to antigens that effectively induce responses either by themselves or together with enhancing materials called "adjuvants." For T cells in particular, antigens and immunogens are not one and the same (Fig. 1). Even preprocessed peptides and MHC-peptide complexes are weak immunogens. This was evident early on in the work of Peter Medawar, the great scientist who discovered the immune basis of transplantation. He spent many years trying to purify functioning transplantation antigens. These efforts were to little avail.

What was not known in Medawar's time is that transplantation antigens

(later shown to be MHC-peptide complexes) become immunogenic when presented by DCs.⁴ In other words, transplantation antigens when presented on many cell types are weak immunogens, but on DCs they become powerful inducers of immunity.⁴ The same is true of peptides that become much more immunogenic when presented on DCs. DCs activate T cells by getting them to divide and express their helper and killer functions. Then the activated T cells interact with other antigen-presenting cells to eliminate the antigen in question. DCs are also called "nature's adjuvant," because prior adjuvants were artificial substances used to enhance immunity. The DC advantage entails a myriad of functions, some of which will be considered below.

Potency of dendritic cells in initiating immunity in tissue culture

What are some specific features of DCs that warrant attention? The first is their potency. Very small numbers of DCs are sufficient to trigger strong T-cell responses in test tubes. Immune assays are generally carried out with impure antigen-presenting cells, applied at a dose of one presenting cell for every T cell, the latter often preactivated. In contrast, roughly one DC per 30-100 T cells is more than sufficient to induce optimal responses, including responses by resting T cells. A single DC can simultaneously activate 10-20 T cells nestled within its sheet-like processes. Therefore, DCs are more than antigen-presenting cells; they are accessories or adjuvants or catalysts for triggering and controlling immunity.

It has always been clear that the accessory function of DCs did not depend exclusively on their capacity to process antigens to form MHC-peptide complexes. This is because the stimuli that were used to define the potency and immune-activating role of DCs did not require that the DCs process an applied antigen. Such stimuli included major transplantation antigens, mitogens, contact allergens, anti-

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T-cell antibodies and superantigens. Furthermore, once resting T cells were activated by DCs, the T cells responded vigorously to antigens presented by other cell types, showing that the latter were not deficient in forming ligands for the antigen receptor on T cells, but instead lacked accessory properties.

The word "accessory" has since been replaced by the terms "professional" and "co-stimulatory," but the basic concept is unchanged by shifting terminology. T cells need stimuli other than their specific trigger or ligand (MHC-peptide complexes) to begin to grow and function, for example, to produce the interleukins and killer molecules mentioned above. DCs are potent in providing the needed accessory or co-stimulatory functions. For example, DCs produce an adhesion molecule called DC-SIGN that binds to a target on resting T cells called ICAM-3,⁵ and DCs express very high levels of a stimulatory molecule called CD86 that binds to CD28 on resting T cells.⁶ These are but two examples of the specialized activities of DCs. These cells do not operate as a single magic bullet.

Position of dendritic cells *in vivo*

Another special feature of DCs is their location and movement in the body. As criteria were developed to identify DCs, it became feasible to go back into the animal and patient to look for the corresponding cells in different tissues. DCs are stationed at surfaces where antigens gain access to the body (Fig. 2, left). The skin and the airway have been the best studied. DCs are found in afferent lymphatic vessels, special channels that allow cells to move from peripheral tissues to lymphoid organs, primarily the T-cell areas (Fig. 2, middle and right). This migration is most readily observed in models of skin transplantation and contact allergy, which are the two most powerful immune responses known.

DC migration is likely to be very important. The body's pool of T cells primarily traffics through the T-cell areas of lymph nodes, rather than

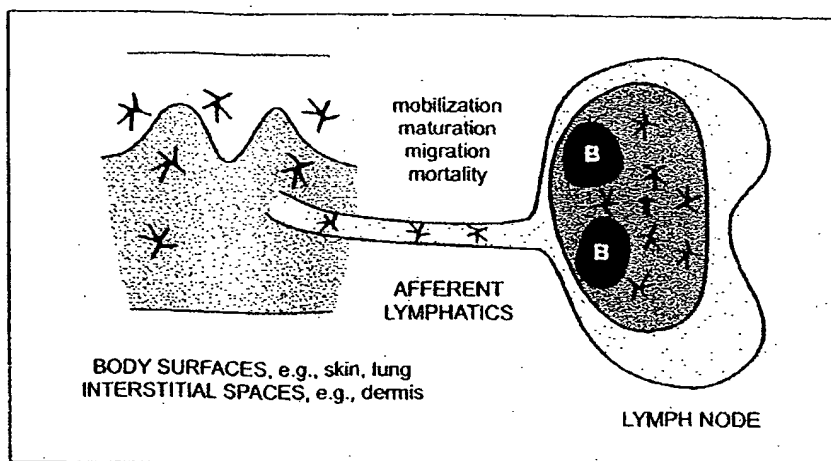


Fig. 2. Distribution of dendritic cells *in situ*. DCs at body surfaces and in solid organs can pick up antigens, move to the lymphoid tissues to find antigen-specific T cells and initiate immunity. Molecular mechanisms are being uncovered that govern the mobilization, maturation, migration and mortality of these DCs. In the lymph node, T lymphocytes are selected for expansion and differentiation into helper and killer T cells. The activated T cells then leave the lymph node to return to the body surface or peripheral organ to eliminate the antigen.

through tissues where antigens are usually deposited. So when DCs capture antigens in the skin, airway or another peripheral tissue, their migration to the T-cell areas gives them a chance to select the corresponding rare specific T cells from the assembled repertoire (Fig. 2). The selected T cells then increase in numbers (clonal expansion) and function, enabling the specific immune response to begin. The initial frequency of T cells that recognize an antigen is very small. Only one in 10,000–100,000 of T cells in the repertoire responds to a specific MHC-peptide complex. Therefore, it is so precise and efficient for DCs to be able to pick up an antigen in the periphery and then initiate the immune response from rare T-cell clones in lymphoid organs.

The events that make up the life history of DCs (Figs. 2 and 3) are now being unraveled in molecular terms. For example, scientists are figuring out how to expand antigen-capturing precursors to DCs using flt3 ligand and granulocyte colony-stimulating factor (G-CSF). Key players for the mobilization of DCs from the periphery to lymph nodes are the multidrug resistance receptors, usually studied for their capacity to mediate resistance to chemotherapeutic agents rather than

movement of DCs. Migration of DCs is controlled by chemokines produced in the lymphatic vessels and lymphoid organs (Fig. 2). These act on DC chemokine receptors to orchestrate their movement to the T-cell areas. Then within the lymphoid tissue, several members of the tumor necrosis factor (TNF) and TNF-receptor families, such as TRANCE and CD40 ligand, trigger DC production of cytokines like interleukin-12. The TNF family also maintains DC viability. Otherwise the cells die within a day or two. Each of these components of DC function provides targets for manipulating immunity.

Priming of T-cell immunity via dendritic cells

Animal studies

During the early research on DCs, several labs administered antigens to experimental animals and then tried to identify the cells that had captured the antigens in a form that was immunogenic. Regardless of the route of antigen administration (blood, muscle, skin, intestine and airway), DCs were the major reservoir of immunogen.

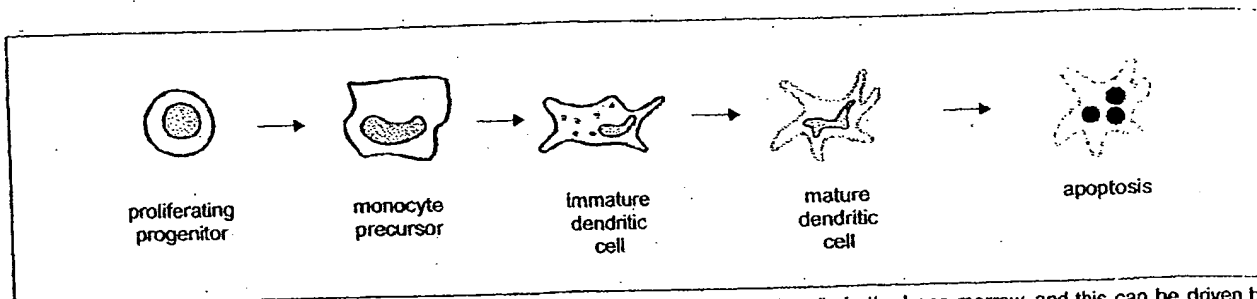


Fig. 3. The life history of dendritic cells. DCs arise from proliferating progenitors, primarily in the bone marrow, and this can be driven by cytokines like flt-3 ligand and G-CSF. Precursors are formed, such as the monocytes in blood, which then give rise to immature DCs. The immature DCs are capable of producing large amounts of antigen-presenting MHC products and capturing antigens. Multidrug resistance receptors are newly recognized players in the mobilization of immature DCs. DCs mature in response to various stimuli such as infection and inflammation, and migrate under the influence of chemokines to the lymphoid tissues. There the DCs die within a day unless their lifespan is prolonged by TNF-family molecules expressed by the activated T cells.

Next, DCs were used as nature's adjuvant to immunize animals. The DCs were taken from mice or rats, exposed to antigens *ex vivo* and injected back into immunologically naive recipients. The animals became immunized to the antigens that had been captured by the DCs, and the immunization took place in the lymph nodes draining the site of DC injection. Genetic proof was provided that the DCs were priming the animal directly and not simply handing off their antigen to other cells.^{7,8}

DC-based immunization is really very different from all prior attempts at cell therapy. Immunology has had extensive experience with "passive immunization," whereby a recipient is given large numbers of cells that are activated prior to injection. It is hard to produce such large numbers of cells, and their lifespan, diversity and efficacy are all finite. In contrast, when relatively small numbers of antigen-charged DCs are used to induce immunity, this produces "active immunization." Now the animals (and patients, see below) can make their own diverse and longer lasting immune response to the antigen-bearing DCs.

Human studies

The above experiments made it clear that DCs, pulsed *ex vivo* with antigens, actively immunized animals and raised the exciting possibility that scientists would be able to induce resis-

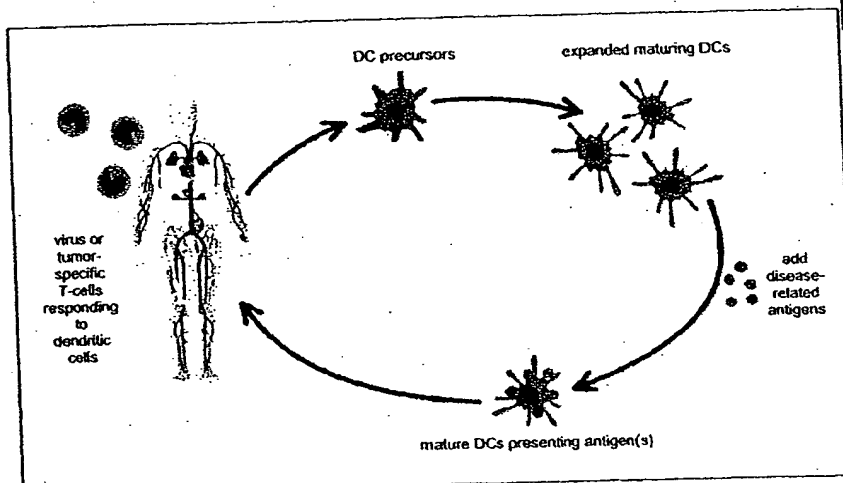


Fig. 4. The use of dendritic cells as adjuvants for enhancing immunity to tumors and infectious agents in humans. This new form of immune therapy begins with the isolation of DC precursors from the patient, usually from blood. The precursors develop *ex vivo* (in relatively simple tissue culture systems) into large numbers of more mature DCs. During this time, the DCs are charged with antigens from the tumor or infection. Then the DCs are reinfused to elicit immunity and thereby resistance to the disease.

tance to tumors, infections and transplants in patients. For example, could one expose patients' DCs *ex vivo* to antigens in their tumors and then reinfuse the antigen-bearing DCs to elicit tumor-specific immunity (Fig. 4)? This approach is actually not terribly complicated, but one first had to overcome a major obstacle and learn to generate large numbers of DCs. These techniques became available in the 1990s. They have energized the field and, accordingly, clinical trials for the immunization of humans against cancer have begun on most continents.

It is evident that DCs can serve as adjuvants for humans, converting antigens into immunogens.^{9,10} Even in advanced cancer, immune responses already have been observed that are similar to or better than immune responses obtained with other approaches. However, this approach is still in its preliminary stages, since a good deal of science remains to be developed. On the one hand, there are critical unknowns in terms of overall DC biology. Many of the clinical studies to date, for example, have overlooked key features that could improve DC function, such as the need for DCs

to be sufficiently mature (see below) to be effective *in vivo*. Also, DC biology has to be placed in the context of specific tumors and pathogens and patients for DC-based therapies to be optimized.

To summarize and further illustrate the role of DCs in the context of human disease (Table I), consider the need to harness T cells to resist tumors and chronic infections. Protein antigens often are known for a tumor-like melanoma, or for a virus like HIV-1 whose genetic sequence has been available for more than 15 years. However, this knowledge about antigens from melanoma and HIV-1 antigens remains to be converted into methods that provide better immunogens either for immune therapy of melanoma or for the design of HIV-1 vaccines. This is because some important facts of immunological life are being overlooked. When antigens are injected, they also need to gain access to the right DCs to become immunogens (Fig. 1).

Delivering antigens to dendritic cells

Broadly speaking, a central goal is to learn how to deliver or "target" antigens to DCs and simultaneously to differentiate or "mature" the cells to their most potent state. These two challenges, antigen targeting and DC maturation, prove to be intertwined.

Targeting means that the antigen should be in a form that the DCs can recognize. Without such recognition, the uptake and subsequent processing of antigen to form MHC-peptide complexes is suboptimal. DCs have a number of special mechanisms for capturing antigens and converting these into MHC-peptide complexes (Table II). For example, DCs have a receptor called DEC-205 whose binding partners or ligands are still unknown. Nonetheless, it is clear that DEC-205 greatly increases the capacity of DCs to form MHC-peptide complexes.¹¹ DCs also carry out a fascinating process called "cross-presentation." DCs can take up dying cells and effi-

ciently extract peptides from them, so antigens "cross" from the dying cell to the DC. The discoverers of this phenomenon called it "resurrecting the dead."¹² Cross-presentation allows DCs to efficiently form MHC-peptide complexes from dead cells in tumors, transplants and tissues under autoimmune attack.

Special uptake and processing mechanisms allow DCs to tailor a protein antigen, as well as the proteins in a complex microbe or tumor cell, into peptides that bind to an individual's MHC products. The latter are exceptionally polymorphic, differing genetically from one individual to another. As a result, the relevant immunizing peptides differ from one individual to another. One reason why peptides are not ideal immunogens is that they must be individualized. DCs, in contrast, can capture antigens with high efficiency and likewise extract peptides that are relevant for any individual.

A second DC advantage is that these cells have the many required accessory or co-stimulatory properties for converting the selected peptides ("antigens") into effective immunogens. A third DC advantage is that these cells position themselves in a way that leads to the identification of rare antigen-reactive T lymphocytes *in vivo* (Fig. 2). DCs thus overcome many of the difficult obstacles in initiating immunity.

In order for an antigen to be a strong immunogen, one needs to provide a stimulus for the final differentiation or maturation of the DCs (Fig. 3). Most DCs in the body are in an immature state and lack many features that lead to a strong T-cell response.

Immature DCs, for example, lack the CD86 and CD40 molecules that greatly boost the DC-T cell interaction. Immature DCs also lack a chemokine receptor called CCR7 that seems very important for proper migration and homing to lymph nodes to start immunity. For cancer immunology, it is unlikely that tumors provide maturation stimuli. Tumors may even block DC maturation induced by other stimuli. Therefore it is important to learn how to deliver tumor cells to DCs and bypass the normal obstacles to effective antitumor immunity.

Surprising recent evidence actually links DC maturation to the efficient formation of MHC-peptide complexes or TCR ligands (Table II). Immature DCs take up antigens, but they do not make abundant MHC-peptide complexes until they receive a maturation stimulus.^{13,14} Maturation also up-regulates CD86 co-stimulators, but the CD86 actually travels together with the TCR ligands to the surface of the DCs. At the DC surface, the MHC molecules and CD86 remain clustered with each other, keeping the machinery for T-cell activation juxtaposed. This phenomenon will help explain the potency of DCs, because TCR ligands and co-stimulators are displayed together on the cell surface and in high levels.

Control points beyond antigen targeting and maturation of DCs

Research on DCs is moving more vigorously, because the cells are more readily available and because their role in the immune system is considered essential. Nonetheless, researchers in this field are just beginning to find ways to manipulate DCs *in situ*. Putting together an antigen that targets

TABLE II: DENDRITIC CELL SPECIALIZATION TO INCREASE MHC-PEPTIDE COMPLEX FORMATION

- Receptors for antigen uptake, e.g., DEC-205
- Processing of dying cells, nonreplicating microbes and immune complexes onto MHC class I ("cross-presentation")
- Regulation of antigen processing by maturation stimuli
- Clustering of T-cell receptor ligands with co-stimulators like CD86

to DCs with a stimulus for DC maturation will be a major step in improving the conversion of antigens into immunogens, as in immune-based therapies against tumors and infectious agents.

Additional challenges and questions are evident:

- How can DC numbers be increased *in situ* and how can active DCs be mobilized to a cancer or site of chronic infection?
- Can DCs induce strong immune memory to make vaccination long lasting and effective (we have only been reviewing the role of DCs in the initiation of immunity)?
- Can DCs change the quality of the immune response? "Quality" refers to recent evidence for different types of DCs, especially a subset that induces Th1-type T cells for resistance to infectious agents and strong memory.
- Is it possible to move beyond DC-based immunization experiments and use DCs to either regulate or tolerize the immune system, as frequently required in transplantation and autoimmune diseases?
- Can DCs influence elements of the immune system other than T cells; for example, B cells and the innate defenses provided by natural killer (NK) and NK-T cells?

The answer to all these questions is a preliminary "yes." As research on DCs expands, more potential functions and more sites for their manipulation are becoming apparent.

Dendritic cells and better control of disease

DCs provide important avenues for the investigation of human disease. Many labs are exploiting DCs to identify antigens relevant for immunity against human pathogens. In these experiments, one introduces complex but clinically important antigens to DCs and then identifies which components are best presented to the immune system. We have recently used this approach to identify previously un-

known immune responses to the Epstein-Barr virus,¹⁵ a virus we all carry that has the potential to cause cancer like Hodgkin's lymphoma. Other laboratories have been using DCs to identify new antigens in other infectious agents, in transplants and in cancers like melanoma.

Investigators are also manipulating DCs *ex vivo* and then reinfusing the cells to identify conditions leading to strong immunity in patients (Fig. 4). In particular, DC-mediated active immunization against cancer is being vigorously pursued, as mentioned above. Instead of manipulating DCs *ex vivo*, a more desirable goal would be able to alter DCs directly *in situ*. Some approaches are under way. An example is the injection of cytokines like flt3 ligand and G-CSF to mobilize various precursor populations of DCs. One should also develop methods to control DC mobilization, migration and maturation. In sum, DCs are allowing scientists to overcome a longstanding obstacle to research in immunology by extending the playing field beyond antigens to immunogens and beyond models to pathogens that cause disease.

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Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor

(heterodimeric lymphokine/T-cell growth factor/lymphokine-activated killer cells/coordinate gene regulation/interleukin-12)

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ABSTRACT Cytotoxic lymphocyte maturation factor (CLMF) is a disulfide-bonded heterodimeric lymphokine that (i) acts as a growth factor for activated T cells independent of interleukin 2 and (ii) synergizes with suboptimal concentrations of interleukin 2 to induce lymphokine-activated killer cells. We now report the cloning and expression of both human CLMF subunit cDNAs from a lymphoblastoid B-cell line, NC-37. The two subunits represent two distinct and unrelated gene products whose mRNAs are coordinately induced upon activation of NC-37 cells. Coexpression of the two subunit cDNAs in COS cells is necessary for the secretion of biologically active CLMF; COS cells transfected with either subunit cDNA alone do not secrete bioactive CLMF. Recombinant CLMF expressed in mammalian cells displays biologic activities essentially identical to natural CLMF, and its activities can be neutralized by monoclonal antibodies prepared against natural CLMF. Since this heterodimeric protein displays the properties of an interleukin, we propose that CLMF be given the designation interleukin 12.

The molecular cloning and expression of recombinant cytokines has made possible both significant advances in our understanding of the molecular basis of immune responses and the development of new approaches to the treatment of disease states. As an example, recombinant interleukin 2 (recombinant IL-2) has been shown to be capable of causing regression of established tumors in both experimental animals (1) and in man (2); however, its clinical use has been associated with significant toxicity (2). One potential approach to improving the therapeutic utility of recombinant cytokines is to use them in combination (3, 4). With this concept in mind, we initiated a search for novel cytokines that would synergize with suboptimal concentrations of recombinant IL-2 to activate cytotoxic lymphocytes *in vitro* and thus might have synergistic immunoenhancing effects when administered together with recombinant IL-2 *in vivo*. This led to the identification of a factor, designated cytotoxic lymphocyte maturation factor (CLMF), that synergized with recombinant IL-2 to facilitate the generation of both cytolytic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells *in vitro* (5, 6). CLMF was subsequently purified to homogeneity from a human lymphoblastoid B-cell line (NC-37) and was shown to be a 75-kDa disulfide-bonded heterodimer composed of two subunits with molecular masses of 40 kDa and 35 kDa (7).[‡] We now report the molecular cloning and expression of CLMF.

MATERIALS AND METHODS

cDNA Cloning. A subline of NC-37 cells selected for its ability to produce high levels of CLMF (7), NC-37.98, was induced with phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 for 16 hr. Poly(A)⁺ RNA was isolated, and random hexamer-primed cDNA libraries were established in phage λ gt10 by standard procedures. Mixed-primer polymerase chain reaction (PCR) using controlled ramp times (8) was performed as follows. PCR primers contained all possible codons and were 14 or 15 nucleotides long (Fig. 1) with a 5' extension of 9 nucleotides containing an *Eco*RI site for subcloning. Degeneracies varied from 1 in 32 to 1 in 4096; 0.5–4 pmol per permutation of forward and reverse primer was used in a 50- to 100- μ l PCR mixture with 40 ng of cDNA made from NC-37.98 cells that had been activated by culture with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml for 16 hr (40-kDa subunit) or with 3 μ g of human genomic DNA (35-kDa subunit). PCR cycling parameters were as follows. Initial denaturation was at 95°C for 7 min. Low-stringency annealing was performed by cooling to 37°C over 2 min, incubating 2 min at 37°C, heating to 72°C over 2.5 min, extending at 72°C for 1.5 min, heating to 95°C over 1 min, and denaturing at 95°C for 1 min. This cycle was repeated once. Thirty standard cycles (40-kDa subunit) or 40 standard cycles (35-kDa subunit) were performed as follows: 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. "Amplicons" of the expected size were gel-purified, subcloned, and sequenced. The 40-kDa subunit cDNAs were isolated by hybridizing the 54-mer amplicon in 5 \times SSC (1 \times SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 20% formamide at 37°C overnight. Filters were washed in 2 \times SSC at 42°C for 30 min and exposed to x-ray film. The 35-kDa subunit cDNAs were isolated by hybridizing the 51-mer amplicon in 5 \times SSC/20% formamide at 37°C overnight. The filters were washed in 2 \times SSC at 40°C for 30 min and exposed to x-ray film. Positive clones were plaque-purified, their inserts were subcloned into the pBluescript plasmid, and their sequences were determined by using Sequenase.

Expression. cDNAs were separately engineered for expression in vectors containing the simian virus 40 early promoter essentially as described (9). COS cells were transfected with both CLMF subunit expression plasmids mixed together or

Abbreviations: CLMF, cytotoxic lymphocyte maturation factor; rCLMF and nCLMF, recombinant and natural CLMFs; CTL, cytolytic T lymphocyte; IL, interleukin; LAK, lymphokine-activated killer; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; n, natural; PCR, polymerase chain reaction.

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[‡]The cDNA sequences reported in this paper have been deposited in the Genbank data base [accession nos. M38443 (35-kDa CLMF subunit) and M38444 (40-kDa CLMF subunit)].

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1  MCPARSLLLV ATLVLDHLS IARNLPVATP DPGMFPC^LHH SQNLLRAVSN
51  MLQKARQ^TLE FYPCTSEEID HEDITKDKTS TVEAC^LPLEL TKNESCLNSR
101  ETSFITNGSC LASRKTSFMM ALCLSSIIYED LKMYQVEFKT MNAKLLMDPK
151  RQIFLDQ^NML AVIDELMQAL NFNSETVPQK SSLEEPDFYK TKIKLC^ILLH
201  AFRIRAVTID RVTSYLNAS

1  MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC
51  DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS
101  LLLLHKKEDG IWSTDILKDQ KEPKNKTFIR CEAKNYSGRF TCWWLTIST
151  DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP
201  AAESLPIEV MVDVHKLKY ENYTSSFFIR DIKPDPPKN LQLKPLKNSR
251  QVEVSWEYPD TWSTPHSYFS LTF^CVQVQVK SKREKKDRVF TDKTSATVIC
301  RKNASISVRA QDRYSSSSWS EWASVPCS

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FIG. 1. Amino acid sequences of the 35-kDa (Upper) and 40-kDa (Lower) CLMF subunits as deduced from the respective cDNAs and shown in single-letter code. Signal peptides are overlined, cysteine residues are marked by a caret, and N-linked glycosylation sites (NXS, NXT, where X is another amino acid) are underlined. The peptide sequences used to generate PCR probes are overlined with arrows indicating the direction of amplification.

with each one separately by the DEAE-dextran method. Twenty-four hours after transfection, the serum-containing medium was replaced with medium containing 1% Nutridoma-SP (Boehringer Mannheim), and supernatant fluids were collected from the cultures after 40 hr. These fluids were stored at 4°C until tested in the bioassays.

General Methods. Standard molecular biological procedures were used as described (10). CLMF bioassays were performed as detailed (7).

Computer Searches. The National Biomedical Research Foundation protein data base (Release 26.0) as well as the Genbank and European Molecular Biology Laboratory databases (Releases 65.0 and 24.0, respectively) were searched for sequences homologous to CLMF cDNAs. The two subunit sequences were compared to each other using the ALIGN program (mutation data matrix, break penalty of 6; see ref. 11).

RESULTS

Partial N-terminal amino acid sequences of the two CLMF subunits (7) were used to generate completely defined 51- to 54-base-pair (bp)-long oligonucleotide probes by means of mixed primer PCR. These probes were used to screen cDNA libraries made from RNA from activated NC-37.98 cells, and cDNAs encoding the two subunits were isolated and characterized. Both cDNAs encode secreted proteins with a classical hydrophobic N-terminal signal peptide immediately followed by the N terminus of the mature protein as determined by protein sequencing (7). Two independent cDNA clones for the 40-kDa subunit were shown to be identical. Both encode the mature 40-kDa subunit that is composed of 306 amino acids (calculated $M_r = 34,699$) and contains 10 cysteine residues and four potential N-linked glycosylation sites (Fig. 1). Two of these sites are within isolated tryptic peptides derived from the purified 40-kDa CLMF subunit protein. Amino acid sequence analysis has shown that Asn-

222 is glycosylated, whereas Asn-125 is not (Fig. 1; F. Podlaski, personal communication). The mature 35-kDa subunit is composed of 197 amino acids (calculated $M_r = 22,513$), with 7 cysteine residues and three potential N-linked glycosylation sites (Fig. 1). When purified CLMF is reduced with 2-mercaptoethanol and analyzed by SDS/PAGE, the 35-kDa subunit appears to be heterogeneous, suggesting that it may be heavily glycosylated (7). Two variants of 35-kDa subunit-encoding cDNAs were isolated. The first type had the sequence shown in Fig. 1. Additional isolates contained what is probably an allelic variation, replacing Thr-213 with a methionine residue.

Computer searches of sequence databases showed that the amino acid sequences of the two subunits are not related to any known protein. The subunit sequences are also not related to each other, since a comparison using the ALIGN program (11) gave a score of 1.27; only scores >3 are considered to indicate significant evolutionary relationship (12). The genes encoding the subunits appear to be unique, since low- and high-stringency hybridizations of genomic blots revealed identical banding patterns (data not shown). RNA blots showed the size of the 40-kDa subunit mRNA to be 2.4 kb, whereas the 35-kDa subunit was encoded by a 1.4-kb transcript (Fig. 2). Expression of the two mRNAs encoding the subunits was coordinately regulated upon induction (Fig. 2). When NC-37.98 cells were activated with PMA and calcium ionophore for 72 hr, mRNA encoding each of the CLMF subunits was minimally detectable at 6 hr after the beginning of induction but was readily detected at 24 hr and continued to accumulate until maximal levels were reached at 72 hr (normalized to GAPDH mRNA levels; see the legend to Fig. 2). In contrast, the mRNA for IL-2 in activated NC-37.98 cells was already at high levels at 6 hr and subsequently decreased, whereas the mRNAs for the low-affinity IL-2 receptor (p55) followed the induction pattern seen for the CLMF subunits. Scanning of RNA blots also revealed that steady-state mRNA levels for the 40-kDa

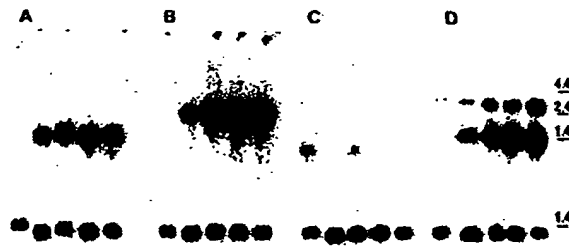


FIG. 2. RNA blots showing the coordinate induction of the 35-kDa (A) and 40-kDa (B) CLMF subunit mRNAs and IL-2 mRNA (C) and its p55 receptor mRNA (D). Poly(A)⁺ RNA (5 μ g) from NC-37.98 cells activated with 10 ng of PMA and 25 ng of calcium ionophore A23187 per ml were loaded in each lane. Lanes from left to right in each panel show RNAs isolated 6, 24, 30, 48, and 72 hr after induction, respectively. (Upper) Four-day exposures. (Lower) Two-hour exposure of the same blots after stripping and rehybridization with a GAPDH probe. Marker sizes are in kb (BRL RNA ladder).

CLMF subunit were severalfold higher than those for the 35-kDa subunit expressed by the same cells. This finding parallels the observation that activated NC-37 cells secrete excess free 40-kDa subunit protein (7). The 3' untranslated sequences of both CLMF subunit mRNAs contain several copies of the octamer motif TTATTTAT (data not shown). This sequence is present in other transiently expressed mRNAs and is involved in regulating mRNA stability (13).

Coexpression of the 40-kDa and 35-kDa CLMF subunit cDNAs in COS cells was required to generate secreted biologically active CLMF (Table 1 and Fig. 3). COS cells transfected with cDNA encoding either the 40-kDa subunit alone or the 35-kDa subunit alone did not secrete biologically active CLMF (Table 1). Mixing media conditioned by COS

cells that had been separately transfected with one or the other of the two CLMF subunit cDNAs also did not give rise to bioactive CLMF (Table 1).

Two types of assays were used to compare rCLMF and nCLMF. The first assay measures the proliferation of phytohemagglutinin (PHA)-activated human peripheral blood lymphocytes, whereas the second assay evaluates the synergy between CLMF and suboptimal concentrations of IL-2 in the generation of LAK cells in hydrocortisone-containing cultures (7). The data in Fig. 3 show that rCLMF as expressed in COS cells and nCLMF as purified from NC-37 cells are essentially identical. Dose-response curves for rCLMF and nCLMF were superimposable in each of the two assays, and rCLMF was neutralized by a monoclonal antibody raised against nCLMF. Conditioned media from cultures of mock-transfected COS cells displayed no activity in these assays (Table 1 and data not shown).

DISCUSSION

In a previous report (7), we described the purification of a heterodimeric cytokine, CLMF, that synergized with low amounts of IL-2 to cause the generation of LAK cells in the presence of hydrocortisone and stimulated the proliferation of activated T cells independent of IL-2. In the present report, we have used the N-terminal amino acid sequence information previously obtained to clone the two subunit cDNAs of CLMF. Protein purification of NC-37 cell line-derived CLMF had shown that the protein was composed of two disulfide-bonded subunits with different N-terminal amino acid sequences (7). However, it was not clear from our previous results whether the two subunits were processed from one common gene product and whether proteolytic posttranslational processing other than signal peptide cleavage was occurring. The molecular cloning and sequencing of

Table 1. Coexpression of both CLMF subunit cDNAs is required for secretion of biologically active CLMF by COS cells

Addition	Conc., units/ml	Dilution	[³ H]Thymidine incorporated by PHA-activated lymphoblasts, mean cpm \pm 1 SEM
Cytokine*			
None	—		11,744 \pm 514
nCLMF	200		68,848 \pm 878
nCLMF	40		48,827 \pm 605
nCLMF	8		26,828 \pm 594
nCLMF	1.6		17,941 \pm 196
Culture fluid from COS cells transfected with			
A. 35-kDa CLMF subunit cDNA		1:20	11,912 \pm 660
		1:100	10,876 \pm 232
B. 40-kDa CLMF subunit cDNA		1:20	11,699 \pm 931
		1:100	11,666 \pm 469
C. 35-kDa + 40-kDa CLMF subunit cDNAs		1:20	58,615 \pm 587
		1:100	38,361 \pm 828
1:1 mix of culture fluids A and B		1:10 [†]	11,544 \pm 483
		1:50	10,503 \pm 259
CM from mock-transfected control [‡]		1:20	11,503 \pm 286
		1:100	10,751 \pm 303

PHA-activated lymphoblasts were prepared from human peripheral blood mononuclear cells as described (7). Lymphoblast proliferation was measured in a 48-hr assay (7) in which 2×10^4 lymphoblasts were incubated in 100- μ l cultures containing the indicated amounts of natural CLMF (nCLMF) or COS cell culture fluids. [³H]Thymidine was added to each culture 18 hr prior to harvest. Conc., concentration.

*nCLMF is purified NC-37-derived CLMF.

[†]1:10 dilution of the 1:1 mixture of culture fluids A and B was equivalent to a 1:20 final dilution of each of the individual culture fluids.

[‡]Conditioned medium (CM) from cultures of mock transfected COS cells.

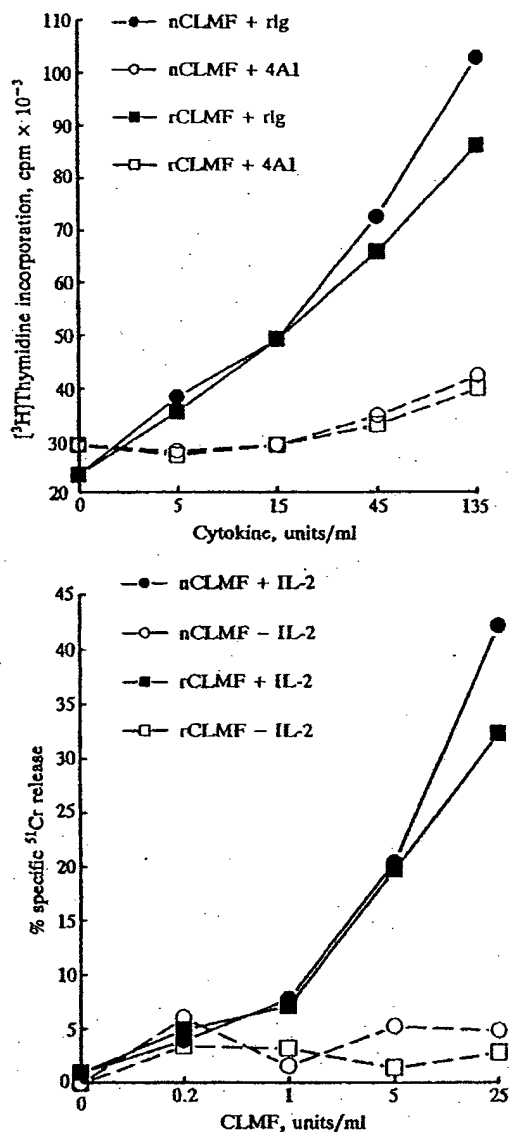


FIG. 3. Comparison of biologic activities of nCLMF (circles) and recombinant CLMF (rCLMF, squares). nCLMF was purified from NC-37 cell-conditioned media; rCLMF was purified from conditioned media from cultures of COS cells transfected with cDNAs encoding the 40-kDa and 35-kDa human CLMF subunits. (Upper) T-cell growth factor assay. The ability of CLMF to stimulate the proliferation of human PHA-activated lymphoblasts in 48-hr cultures was assayed as described (7). CLMF preparations were mixed with neutralizing rat monoclonal anti-human CLMF antibody 4A1 (ref. 7; open symbols) or with normal rat IgG (Sigma; rlg, closed symbols) at a final concentration of 20 μ g of IgG/ml and were incubated for 30 min at 37°C prior to addition of PHA blasts. All values are means of triplicate determinations. (Lower) LAK cell induction assay. The ability of CLMF, alone or in combination with recombinant IL-2, to induce the generation of LAK cells in 4-day cultures was assessed as described (7). Low-density peripheral blood lymphocytes were incubated in the presence of various amounts of nCLMF or rCLMF with (closed symbols) or without (open symbols) recombinant IL-2 at 7.5 units/ml. Units of CLMF activity were based on previous titrations in the T-cell growth factor assay. Hydrocortisone sodium succinate (Sigma) was included at a concentration of 0.1 mM to minimize triggering of endogenous cytokine cascades. Lysis of ⁵¹Cr-labeled Daudi cells was assessed at an effector/target ratio of 5:1. The data shown represent the means of quadruplicate determinations. The spontaneous ⁵¹Cr release was 20%.

the corresponding cDNAs now has demonstrated that there is no common precursor for the two CLMF subunits; rather, they are encoded by completely different genes. The predicted and actual amino acid composition for the two subunits are strikingly similar; differences in predicted versus actual molecular weights are accounted for by glycosylation (F. Podlaski, personal communication). Thus, the only major posttranslational proteolytic event that appears to take place in the maturation of the CLMF subunits is signal peptide cleavage.

The kinetics of expression of the individual CLMF subunit mRNAs were examined and compared to the expression of mRNAs for IL-2 and the IL-2 receptor p55. Previously it had been observed that NC-37 cells, like certain murine (14) and marmoset (15) B-cell lines, secreted IL-2 when activated (M.K.G., unpublished results). RNA blots demonstrated that upon activation of NC-37 cells, both CLMF subunit mRNAs were coordinately induced with kinetics similar to the IL-2 receptor (p55) mRNAs. On the other hand, IL-2 mRNA levels peaked much earlier. Similar differences in induction kinetics were also seen at the level of IL-2 and CLMF bioactivity secreted from NC-37 cells (M.K.G., unpublished data). These kinetic differences are consistent with our previous observation that in a cytolytic lymphocyte response, IL-2 appears to act earlier than CLMF (5).

Transfection studies with COS cells established that only coexpression of both subunit cDNAs gives rise to secreted bioactive CLMF. Thus, it appears that the two proteins have to interact within the endoplasmic reticulum to assemble properly into bioactive secreted CLMF. By comparing the activity of rCLMF to that of nCLMF in the T-cell growth factor and LAK cell induction assays (Fig. 3) and assuming that the specific activity of rCLMF is similar to that of nCLMF [8×10^7 units/mg (7)], we estimate that the amount of rCLMF heterodimer produced in these experiments was 5–50 ng/ml. The finding that COS cells, which are fibroblast-like cells, are able to assemble correctly the two CLMF subunits to form bioactive CLMF indicates that this secretion and processing pattern is not limited to cells of the lymphoid lineage.

Western blot analysis using an anti-CLMF antibody specific for the 40-kDa subunit has allowed confirmation that (i) COS cells transfected with both CLMF subunit cDNAs secrete CLMF with the expected heterodimeric structure and (ii) COS cells transfected with the 40-kDa subunit cDNA alone secrete that subunit (F. Podlaski, personal communication). Since no bioactivity was detected in media conditioned by COS cells transfected with only the 40-kDa subunit, that subunit by itself appears either to have a much reduced specific activity compared with heterodimeric CLMF or to be completely inactive.

Because of the lack of a high-affinity antibody specific for the 35-kDa subunit, we have not yet been able to determine definitively whether COS cells transfected with only the 35-kDa subunit cDNA secrete that subunit. Since no bioactivity was detected in the media, secretion of a bioactive 35-kDa subunit by itself could be very inefficient; alternatively, similar to the 40-kDa subunit, the protein could be much less active or inactive altogether. Intracellular 35-kDa protein in the absence of the other subunit could be inherently unstable; there is precedence for this phenomenon, since it has been reported that 90% of the β chains of lutropin (LH), when expressed in the absence of α chains, are retained in the endoplasmic reticulum and are slowly degraded (16). Simple mixing of media conditioned by COS cells transfected separately with either one of the two CLMF subunit cDNAs did not yield bioactive CLMF. One possible explanation would be that the cells do not secrete the 35-kDa CLMF subunit by itself. More likely, our experimental conditions did not allow proper heterodimer formation. One would expect that only

carefully controlled renaturation and oxidation conditions would allow the disulfide bond formation required for generation of bioactive CLMF.

Normal human peripheral blood lymphocytes under the appropriate induction conditions produce both CLMF subunit mRNAs and secrete the active protein (N.N. and M.K.G., unpublished data). There is some evidence suggesting that CLMF is produced predominantly by B cells. In preliminary experiments, B-cell mitogens have appeared to be more effective than T-cell mitogens in eliciting CLMF production from peripheral blood lymphocytes (M.K.G., unpublished results). When screening human cell lines for their ability to produce CLMF activity (7), we observed that four of eight B-cell lines tested produced CLMF after activation with PMA and calcium ionophore, whereas none of five T-cell lines produced CLMF. Nevertheless, three of these T-cell lines secreted large amounts of IL-2 and tumor necrosis factor activity after activation (M.K.G., unpublished results). Likewise, natural killer cell stimulatory factor (NKSf), a heterodimeric cytokine similar or identical to CLMF, was isolated from RPMI 8866 lymphoblastoid B cells (17). A recent report (18) has indicated that B lymphocytes can secrete a cytokine(s) distinct from IL-2 that facilitates virus-specific cytolytic T-lymphocyte responses. It is possible that CLMF may have been the cytokine active in those studies. Thus, although B lymphocytes have not traditionally been viewed as cytokine-producing helper cells, it is conceivable that CLMF production constitutes a novel mechanism whereby B lymphocytes contribute to the amplification of T-lymphocyte responses. In addition to the biologic activities described in this report, CLMF by itself has been shown (i) to activate NK cells in an 18–22 hr assay, (ii) to facilitate the generation of specific allogeneic CTL responses, and (iii) to stimulate the secretion of γ interferon by resting peripheral blood lymphocytes (M.K.G., unpublished results). It can also synergize with low concentrations of recombinant IL-2 in the latter two assays and in causing the proliferation of resting peripheral blood lymphocytes. In view of its production by peripheral blood lymphocytes and its diverse actions on lymphoid cells, it appears that CLMF constitutes a new interleukin. We propose that CLMF be

given the provisional designation IL-12. The availability of recombinant CLMF will now make possible a broader and more detailed characterization of its biology.

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Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8⁺ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

Patients and Methods: Patients were HLA-A2-positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

Results: Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a

complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8⁺ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma-producing T cells against Melan-A ($P = .015$) after vaccination, but not against an Epstein-Barr virus control peptide ($P = .86$). There was a correlation between the magnitude of the increase in Melan-A-specific cells and clinical response ($P = .046$).

Conclusion: This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

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MOST MELANOMA tumors express antigens that can be recognized by CD8⁺ T cells.^{1,2} Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.³ Specific CD8⁺ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.⁴

The optimal immunization strategy for inducing tumor antigen-specific CD8⁺ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived^{5,6} and bone marrow-derived⁷ dendritic cells (DCs) have been loaded with

melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN- γ)-producing cytolytic effector cells.⁸⁻¹⁰ Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors^{11,12} and provision of exogenous IL-12, either alone¹³ or combined with tumor antigen-based vaccines,¹⁴⁻¹⁹ can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.²⁰ The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.²¹ The effective range of doses indicated that a straight dose of 4 μ g might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1³ peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2-positive patients with

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advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.⁴ The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2-positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥ 70 , and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophyton*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.²² Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophyton*) by DTH skin testing.

RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.²¹ Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide-stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare CD8⁺ and CD8⁻ fractions for subsequent correlative immunologic studies. The Melan-A₂₇₋₃₅ peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at

Table 1. Patient Characteristics

Patient Characteristic	Patients (n = 20)	
	No.	%
Age, years		
Median		58
Range		35-79
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2		None
≥ 3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy		None
Adjuvant IFN- α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN- α , interferon alpha-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

-80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 μ mol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 μ mol/L; target number of PBMCs, 10^6) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100×10^6 .

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μ g under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μ g) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as $\geq 50\%$ reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

CD8⁺ T-Cell Preparation

CD8⁺ and CD8⁻ fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. CD8⁺ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound CD8⁻ fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific CD8⁺ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, CD8⁻ cells were thawed from each time point and pooled, pulsed with 50 $\mu\text{mol/L}$ Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with beta₂-microglobulin, irradiated (3,000 rad), washed, and plated at 2×10^6 cells/well in 24-well plates. CD8⁺ T cells were thawed and cultured with the irradiated CD8⁻ cells at 4×10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A-pulsed irradiated CD8⁻ cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 $\mu\text{g/mL}$ of antihuman IFN- γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and CD8⁺ T cells, either freshly thawed at 5×10^4 cells/well or after in vitro expansion at 5×10^5 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing-deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50 $\mu\text{mol/L}$ peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of CD8⁺ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 $\mu\text{g/mL}$) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN- γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin-alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN- γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

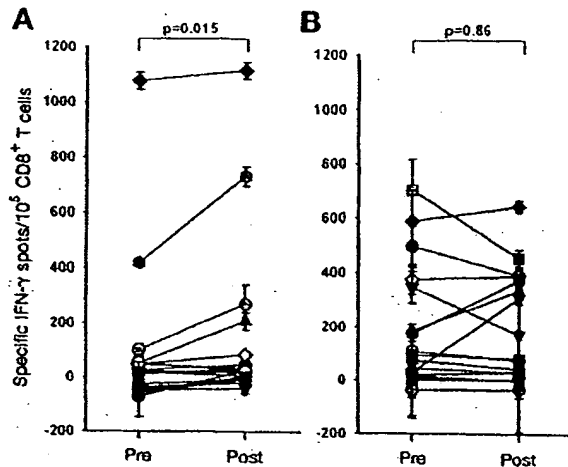


Fig 1. Interferon gamma ELISpot frequencies by CD8⁺ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired *t* test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN- γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved CD8⁺ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A-specific CD8⁺ T cells before vaccination, with as high as 1% of CD8⁺ cells responding to this peptide. These T cells were functional because they produced IFN- γ . The majority of patients showed an increase in the frequency of Melan-A-specific cells after immunization ($P = .015$). In contrast, the frequencies of specific CD8⁺ T cells responding to the EBV peptide did not vary significantly overall ($P = .86$). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A-specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A-specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16 , indicating that a greater absolute increase in Melan-A-specific T cells was associated with tumor regression ($P = .046$).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known

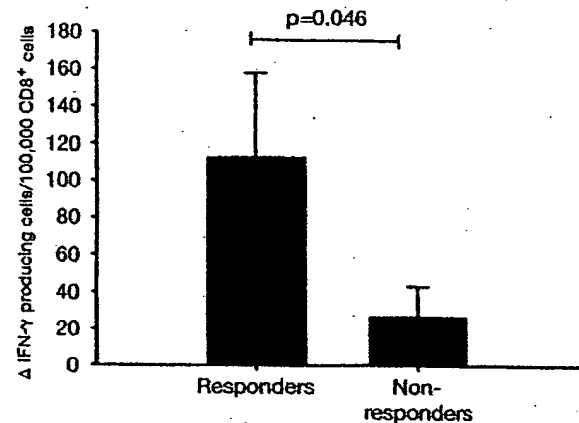


Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired *t* test.

negative prognostic factor,²³ survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ($P = .005$). In addition, the median survival for patients who experienced a significant increase in Melan-A-specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A-specific cells (Fig 3C; $P = .120$).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A-specific CD8⁺ T cells before immunization correlated with either outcome. The median pretreatment Melan-A-specific T cell frequency was 23 in clinical nonresponders and -26 in responders. To increase the sensitivity of the assay to detect Melan-A-specific T cells, an *in vitro* expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A-specific T cell frequencies after *in vitro* expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A-specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A-negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

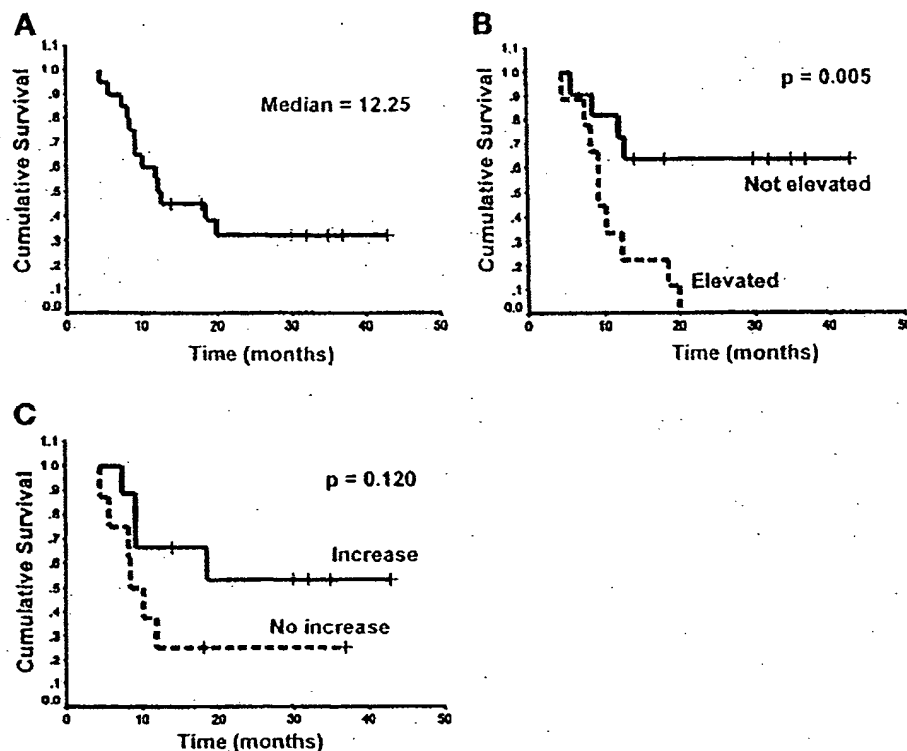


Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing CD8⁺ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2-positive patients with advanced melanoma. We observed a significant increase in Melan-A-specific IFN- γ -producing CD8⁺ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results

support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.²⁴

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved CD8⁺ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A-reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified CD8⁺ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of CD8⁺ T cells, CD4⁺ T cells, and natural killer T cells that could have produced IFN- γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A-specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pre†	.290	.644
Strong Melan-A pre-Rx‡	.644	.481
Increased Melan-A post versus pre†	.046	.120
Strong in vitro expansion of Melan-AS	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 CD8⁺ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 CD8⁺ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 CD8⁺ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous CD8⁺ cells and IL-2.

PBMC + rhIL-12 and in other trials^{21,25} in which in vitro expansion had been performed before assessment of IFN- γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells in vivo.

High frequencies of Melan-A-specific, IFN- γ -producing CD8⁺ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A-specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,²⁶ those cells had a naïve surface phenotype and did not produce high levels of IFN- γ . What did correlate with clinical response in our current study is a meaningful increase in Melan-A-specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 CD8⁺ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in

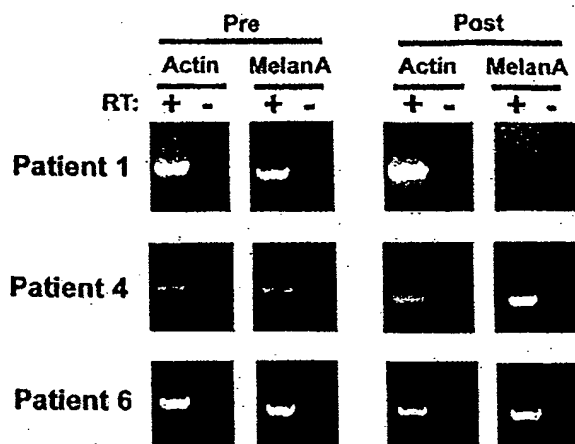


Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

T-cell frequencies using standard assays have been observed in other studies.²⁷

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A-specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies²⁸ and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.^{29,30} Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells in vivo.

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Exh E

Vaccination with Mage-3A1 Peptide-pulsed Mature, Monocyte-derived Dendritic Cells Expands Specific Cytotoxic T Cells and Induces Regression of Some Metastases in Advanced Stage IV Melanoma

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Summary

Dendritic cells (DCs) are considered to be promising adjuvants for inducing immunity to cancer. We used mature, monocyte-derived DCs to elicit resistance to malignant melanoma. The DCs were pulsed with Mage-3A1 tumor peptide and a recall antigen, tetanus toxoid or tuberculin. 11 far advanced stage IV melanoma patients, who were progressive despite standard chemotherapy, received five DC vaccinations at 14-d intervals. The first three vaccinations were administered into the skin, 3×10^6 DCs each subcutaneously and intradermally, followed by two intravenous injections of 6×10^6 and 12×10^6 DCs, respectively. Only minor (less than or equal to grade II) side effects were observed. Immunity to the recall antigen was boosted. Significant expansions of Mage-3A1-specific CD8⁺ cytotoxic T lymphocyte (CTL) precursors were induced in 8/11 patients. Curiously, these immune responses often declined after the intravenous vaccinations. Regressions of individual metastases (skin, lymph node, lung, and liver) were evident in 6/11 patients. Resolution of skin metastases in two of the patients was accompanied by erythema and CD8⁺ T cell infiltration, whereas nonregressing lesions lacked CD8⁺ T cells as well as Mage-3 mRNA expression. This study proves the principle that DC "vaccines" can frequently expand tumor-specific CTLs and elicit regressions even in advanced cancer and, in addition, provides evidence for an active CD8⁺ CTL-tumor cell interaction in situ as well as escape by lack of tumor antigen expression.

Key words: dendritic cells • vaccination • active immunotherapy • melanoma • cytotoxic T lymphocytes

It is now established that the immune system has cells, particularly CD8⁺ CTLs, that can recognize tumor antigens and kill tumors (1, 2). Nevertheless, a major problem is that these T cells are either not induced or only weakly induced, i.e., the T cells are not evident in the systemic circulation. One possibility is that there is inadequate tumor antigen presentation by dendritic cells (DCs),¹ "nature's adjuvant" for eliciting T cell immunity (3). Another is that

tumor-reactive T cells are tolerized by the tumors (1, 4). Melanoma provides a compelling setting in which to pursue a current goal of cancer immunotherapy, the generation of stronger tumor-specific T cell immunity, particularly with CTLs (4). The majority of tumor antigens identified so far are expressed by melanomas (2). Limited antimelanoma CTL responses have been detected (5), and infusions of IL-2 expanded killer cells can lead to rejection of melanoma (6).

Conventional adjuvants promote antibody rather than CTL responses. Therefore, several novel strategies are being explored to induce tumor-specific T cell immunity. DC vaccination is one of these (3). Immature DCs capture

¹Abbreviations used in this paper: CNS, central nervous system; DCs, dendritic cells; DTH, delayed-type hypersensitivity; MCM, monocyte-conditioned medium; RT, reverse transcriptase; TT, tetanus toxoid.

antigens but lack full T cell-stimulatory activity (7). In the presence of appropriate stimuli, such as inflammatory cytokines, the DCs mature. DCs upregulate T cell adhesion and costimulatory molecules as well as select chemokine receptors that guide DC migration into lymphoid organs for priming of antigen-specific T cells. The use of DCs as adjuvants is supported by many animal experiments with primarily mature DCs (3, 8). These studies have shown that the injection of tumor antigen-loaded DCs reliably induces tumor-specific CTL responses, tumor resistance, and in some cases, regression of metastases (3, 8). In the few pilot trials reported so far for humans, immature DCs have been employed (9-11). Scattered tumor responses are reported, but evidence for the induction of tumor-specific CTLs by DC vaccination has not been shown.

We have developed a technique to generate large numbers of homogenous populations of mature and stable DCs from monocytes in the absence of nonhuman proteins (12, 13). We are now exploring the use of these DCs as vaccine adjuvants in humans. Here we provide the proof of the principle by demonstrating that three intracutaneous injections of Mage-3A1 peptide-pulsed mature DCs reliably enhance Mage-3A1-specific CD8⁺ and recall CD4⁺ T cell immunity in heavily pretreated, progressive stage IV melanoma patients with large tumor loads. Expansions of Mage-3A1-specific CTL responses have not been previously detected after Mage-3A1 peptide vaccination in less advanced melanoma patients (14), underscoring the potent adjuvant properties of DCs. As regressions of metastases also occurred upon DC-mediated immunization and were accompanied by CD8⁺ T cell infiltration, we propose that the induced Mage-3A1-specific CTLs are active *in vivo*.

Materials and Methods

Patient Eligibility Criteria

Patients were eligible if they suffered from stage IV (i.e., distant metastases) cutaneous malignant melanoma (1988 American Joint Committee on Cancer/Union Internationale Contre Cancer pTNM staging system) that was not curable by resection and was progressive despite chemo(immuno)therapy. Further inclusion criteria were an expected survival ≥ 4 mo, Karnofsky index $\geq 60\%$, age ≥ 18 yr, measurable disease, HLA-A1 positivity, expression of Mage-3 gene shown by reverse transcriptase (RT)-PCR in at least one excised metastasis, and no systemic chemo-, radio-, or immunotherapy within 4 wk (6 wk in the case of nitrosurea drugs) preceding the first DC vaccination. A positive skin test to recall antigens was not required. Important exclusion criteria were active central nervous system (CNS) metastasis, any significant psychiatric abnormality, severely impaired organ function (hematological, renal, liver), active autoimmune disease (except vitiligo), previous splenectomy or radiation therapy to the spleen, organ allografts, evidence for another active malignant neoplasm, pregnancy, lactation, or participation (or intent to participate) in any other clinical trial. Concomitant treatment (chemo- or immunotherapy, corticosteroids, investigational drugs, paramedical substances) was prohibited. Palliative radiation or surgical therapy of selected metastases and certain medications (acetaminophen/paracetamol, nonsteroidal anti-inflammatory drugs, opiates) to control symptoms were allowed.

Clinical Protocol and Study Design

The study was performed at the Departments of Dermatology in Erlangen, Würzburg, and Mainz, Germany according to standards of Good Clinical Practice for Trials on Medicinal Products in the European Community. The protocol was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research (New York, NY) and performed under supervision of its Office of Clinical Trials Management as study LUD #97-001. The protocol was also approved by the ethics committees of the involved study centers.

The study design is shown in Table II. All patients gave written informed consent before undergoing a screening evaluation to determine their eligibility. Extensive clinical and laboratory assessments were conducted at visits 1, 5, and 8 (Table II) and consisted of a complete physical examination, staging procedures, and standard laboratory values as well as special ones (pregnancy test, free testosterone in males, autoantibody profile, and antibodies to HIV-1/2, human T cell lymphotropic virus type I, hepatitis B virus, and hepatitis C virus). Patients were hospitalized and examined the day before each vaccination and were monitored for 48 h after the DC injections. Adverse events and changes in laboratory values were graded on a scale derived from the Common Toxicity Criteria of the National Cancer Institute, National Institutes of Health, Bethesda, MD.

Production of the DC Vaccine

During prestudy screening, we tested a small amount of fresh blood to verify that appropriate numbers of mature DCs could be generated from the patient's monocytes (12). Sufficient DC numbers could be successfully generated in all patients, but in some patients the test generation revealed that TNF- α had to be added to assure full maturation. To avoid repetitive blood drawings, we performed a single leukapheresis during visit 2 to generate DCs as described (13). In short, PBMCs from the leukapheresis ($\geq 10^{10}$ nucleated cells) were isolated on LymphoprepTM (Nycomed Pharma) and divided into three fractions. The first fraction of 10^9 PBMCs was cultured on bacteriological petri dishes (Cat. #1005; Falcon Labware) coated with human Ig (100 μ g/ml; SandoglobulinTM; Sandoz GmbH) in complete RPMI 1640 medium (BioWhittaker) supplemented with 20 μ g/ml gentamicin (Refobacin-10; Merck), 2 mM glutamine (BioWhittaker), and 1% heat-inactivated human plasma for 24 h to generate monocyte-conditioned medium (MCM) for later use as the DC maturation stimulus. The second fraction of 3×10^8 PBMCs was used for the generation of DCs for vaccination 1 and delayed-type hypersensitivity (DTH) test I. Adherent monocytes were cultured in 1,000 U/ml GM-CSF (10×10^7 U/mg; LeukomaxTM; Novartis) and 800 U/ml IL-4 (purity $>98\%$; 4.1×10^7 U/mg in a bioassay using proliferation of human IL-4R⁺ CTLL; CellGenix; expressed in *Escherichia coli* and produced under good laboratory practice conditions but verified for good-manufacturing practice [GMP] safety and purity criteria by us) for 6 d, and then MCM was added to mature the DCs. MCM was supplemented in patients 04, 06, 09, 11, and 12 with 10 ng/ml GMP-rhu TNF- α (purity $>99\%$; 5×10^7 U/mg in a bioassay using murine L-M cells; a gift of Dr. G.R. Adolf, Boehringer Ingelheim Austria, Vienna, Austria) to assure full maturation of DCs. Mature DCs were harvested on day 7. The third fraction of PBMCs was frozen in aliquots and stored in the gas phase of liquid nitrogen to generate DCs for later vaccinations and DTH tests.

DCs for vaccinations were pulsed with the Mage-3A1 peptide (15) (EVDPIGHLY, synthesized at GMP quality by Clinalfa) as tumor antigen, and as a recall antigen and positive control, tetanus toxoid (TT) or tuberculin (if at visit 1 the DTH to TT in the

Multitest Merieux was >10 mm; both purchased from the Bacterial Vaccines Department of the Statens Serum Institute, Copenhagen, Denmark). The recall antigen was added at 10 µg/ml for the last 24 h, and the Mage-3A1 peptide was added at 10 µM directly to the cultures for the last 8 h (if immunity to recall antigens was strongly boosted, the dose of recall antigen was reduced to 1.0 or 0.1 µg/ml or was omitted for the intravenous DC injections to avoid a cytokine release syndrome). On day 7, mature DCs were harvested, resuspended in complete medium, washed, and pulsed once more with Mage-3A1 peptide (now at 30 µM) for 60 min at 37°C. DCs were finally washed and resuspended in PBS (GMP quality PBS; BioWhittaker) for injection. DCs to be used for Mage-3A1 DTH tests were pulsed with Mage-3A1 (but no recall antigen); DCs that served as negative control in the DTH tests were not pulsed at all. An aliquot of the DCs to be used for vaccinations was analyzed as described (13) to assure that functionally active and mature DCs were generated. The features of the DCs are described in Results. Release criteria were typical morphology (>95% nonadherent veiled cells) and phenotype (>95% HLA-DR⁺CD86⁺CD40⁺CD25⁺CD14⁻ and >65% homogeneously CD83⁺).

Immunization Schedule

A total of five vaccinations (three into the skin followed by two intravenously) with antigen-pulsed DCs were given at 14-d intervals (Table II). This design was chosen to explore the toxicity and efficacy of various routes in this trial. For vaccinations 1–3, 3×10^6 DCs were given subcutaneously at two sites (1.5×10^6 DCs in 500 µl PBS per site) and 3×10^6 intradermally at 10 sites (3×10^5 DCs in 100 µl PBS per site). The injection sites were the ventromedial regions of the upper arms and the thighs close to the regional lymph nodes and were rotated clockwise. Limbs where draining lymph nodes had been removed and/or irradiated were excluded. For intravenous vaccinations 4 and 5, a total of 6 and 12×10^6 antigen-pulsed DCs (resuspended in 25 or 50 ml PBS plus 1% autologous plasma) was administered over 5 and 10 min, respectively. Premedication with an antipyretic (500 mg acetaminophen/paracetamol p.o.) and an antihistamine (2.68 mg clemastinhydrogenfumarat i.v.) was given 30 min before intravenous DC vaccination.

Evaluation of Immune Status

Recall Antigen-specific Proliferation and Cytokine Production. PBMCs were cultured in triplicate at two dose levels (3×10^4 and 1×10^5 PBMCs/well) plus or minus TT or tuberculin (at 0.1, 1, and 10 µg/ml) and pulsed on day 5 with [³H]thymidine for 12 h. In all cases, the highest cpm's were obtained with the highest doses of PBMCs and antigen and are shown in Fig. 2. IL-4 and IFN-γ levels were measured in culture media by ELISA (Endogen, Inc.). In a separate plate, staphylococcal enterotoxin (SEA; Serva) was added at 0.5, 1, and 5 ng/ml, and proliferation was assessed after 3 d to provide a positive control for helper T cell viability and responsiveness.

Enzyme-linked Immunospot Assay for IFN-γ Release from Single Antigen-specific T Cells. To quantitate antigen-specific, IFN-γ-releasing, Mage-3A1-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (16). PBMCs (10^5 and 5×10^5 /well) or in some cases CD8⁺ or CD4⁺ T cells (isolated by MACS[™] according to the manufacturer, Miltenyi Biotec) were added in triplicate to nitrocellulose-bottomed 96-well plates (MAHA S4510; Millipore Corp.) precoated with the primary anti-IFN-γ mAb (1-D1K; Mabtech) in 50 µl ELISPOT

medium (RPMI 1640 and 5% heat-inactivated human serum) per well. For the detection of Mage-3A1-reactive T cells, the APCs were irradiated T2A1 cells (provided by P. van der Bruggen, Ludwig Institute of Cancer Research, Brussels, Belgium) pulsed with MHC class I-restricted peptides (Mage-3A1 peptide and the HIV-1 p17-derived negative control peptide GSEELRSLY) added at 7.5×10^4 /well (final volume 100 µl/well). After incubation for 20 h, wells were washed six times, incubated with biotinylated second mAb to IFN-γ (7-B6-1; Mabtech) for 2 h, washed, and stained with Vectastain Elite kit (Vector Labs.). For detection of TT-reactive T cells, TT was added at 10 µg/ml directly to the PBMCs (1 or 5×10^5 PBMCs/flat-bottomed 96-well plate). Assays were performed on fresh PBMCs. Spots were evaluated and counted using a special computer-assisted video imaging analysis system (Carl Zeiss Vision) as described (16).

Semiquantitative Assessment of CTL Precursors. The multiple microculture method developed by Romero et al. (17) was used to obtain a semiquantitative assessment of CTLp (precursors) specific for Mage-3A1 peptide. Aliquots of frozen PMBCs were thawed and assayed together. CD8⁺ T cells were isolated with magnetic microbeads (MACS[™] separation columns; Miltenyi Biotec) and seeded at 10^4 /well in 96-well round-bottomed plates in RPMI 1640 with 10% heat-inactivated human serum. The CD8⁺ PBMCs were pulsed with peptide Mage-3A1 or the influenza PB1 control peptide VSDGCPNLY (10 µg/ml; 30 min at room temperature), irradiated (30 Gy from a cesium source), and added as an APC population at 10^5 /well together with IL-2 (10 IU/ml final) and IL-7 (10 ng/ml final) in a total volume of 200 µl/well. On day 7, 100 µl fresh medium was substituted, and peptide Mage-3A1 or PB1 (1 µg/ml final) and IL-2 (10 U/ml) was added. On day 12, each microwell was divided into three equal samples to test cytolytic activity in a standard 4-h ⁵¹Cr-release assay on peptide-pulsed (10 µg/ml for 1 h at 37°C) T2A1 cells, nonpulsed T2A1 cells, and K562 target cells, respectively. All of the assays were performed with an 80-fold excess of nonlabeled K562 to block NK activity. Microwells were scored positive if lysis of T2A1 targets with peptide minus lysis without peptide was ≥12% and this specific lysis was greater than or equal to twice the lysis of T2A1 targets without peptide plus six as described (18). We aimed at testing 30 microwells of 10^4 CD8⁺ T cells. Therefore, 1/30 positive wells equals at least one CTLp in 3×10^5 (i.e., 30 wells at 10^4 CTLp per well) CD8⁺ T cells (corresponding to $\sim 3 \times 10^6$ PBMCs).

DTH. DTH to Mage-3A1 peptide was assessed by intradermal injection at two sites of each 3×10^5 Mage-3A1 peptide-loaded DC in 0.1 ml PBS. Negative controls were nonpulsed autologous DCs in 0.1 ml PBS and 0.1 ml PBS. DTH to seven common recall antigens (Multitest Merieux) including TT and tuberculin was performed on visits 1, 5, and 8 (Table II).

Assessment and Analysis of Tumor Tissue

For recruitment into the study, Mage-3 gene expression in at least one metastatic deposit had to be demonstrated by RT-PCR as described (14). Accessible superficial skin metastases were removed whenever possible after the vaccinations and subjected to Mage-3 RT-PCR as well as routine histology and immunohistology (to characterize cellular infiltrates).

Statistical Analysis

For analysis of the immune response, pre- and postimmunization values were compared by paired *t* test after logarithmic transformation of the data. Significance was set at *P* < 0.05.

Results

Patient Characteristics

All 13 patients were HLA-A1⁺, had proven Mage-3 mRNA expression in at least one excised metastasis, and suffered from advanced stage IV melanoma, i.e., distant metastases that were progressive despite chemotherapy and, in some cases, chemoimmunotherapy (Table I). We offered DCs to all patients who fulfilled the inclusion and exclusion criteria, i.e., we did not select for subsets of patients. Two patients (numbers 01 and 03) succumbed to melanoma after two and three vaccinations, respectively. 11 patients received all five planned DC vaccinations in 14-d intervals (Table II) and were thus fully evaluable.

Quality of the Vaccine

All vaccine preparations were highly enriched in mature DCs. More than 95% of the cells were large and veiled in

appearance, expressed a characteristic phenotype by flow cytometry (HLA-DR⁺⁺⁺CD86⁺⁺⁺CD40⁺CD25⁺CD14⁻), and acted as strong stimulators of an MLR at DC/T cell ratios of $\leq 1:300$ (13). Most (mean 80%) expressed the CD83 mature DC marker (19). These features were stable upon removal of cytokines and culture for one to two more days (13). The DCs were pulsed with Mage-3A1 peptide as a tumor antigen and TT or tuberculin as a recall antigen. The latter were internal controls for immunization and possibly provided help for CTL responses (20).

Toxicity

No major (above grade II) toxicity or severe side effects were observed in any patient, including the two patients who were not fully evaluable. We noticed, however, local reactions (erythema, induration, pruritus) at the intracuta-

Table I. Patients' Characteristics, Status before DC Vaccination, and Response to DC Vaccination

Patient code	Sex-Age	Onset stage IV	Previous therapy	Metastases at study entry*							Clinical Response	Survival
				regional		distant					14 days after the 5 th vaccination	
				skin	LN	Skin	LN	Lung	Liver	Other		
Patients with objective tumor regression												
04	M48	1/98	PCI				3/5	10/30		CNS: 2/12	complete regression of all but 1 lung metastasis, overall progression	10 + >9
06	F61	10/97	CI			3/5		10/20	2/10		complete regression* of 1 lung + 4 s.c.* metastases, overall progression	6 + >16
07	F48	6/97	C			1/1				brain: 1/30 bone: 3/70	complete regression* of 1 lung* + 2 s.c.* metastases, overall progression	13 + 12†
08	M67	11/97	PC		2/5		10/30	2/20	2/30		complete regression* of lung + liver + 4 s.c.* metastases, overall progression**	8 + 3†
09	F43	5/98	C					1/28		metast.: 1/45 bone: 2/30	Partial regression of 1 lung metastasis, overall progression	4 + >11
12	M54	9/98	CI			2/5	10/30	10/20			partial regression of axillary LN metastases, overall progression	26 + >9
Patients without objective tumor regression												
02	F73	5/98	PCI	50/40			1/28	1/10	1/35	pancr.: 1/10	continuous progression	18 + 5†
05	F49	10/97	CI		1/10		2/15	1/40			continuous progression	5 + >17
10	M62	8/98	C	6/70		1/10					continuous progression	1 + 6†
11	F72	7/98	C		10/25	2/11		3/12		bone: 1/10	continuous progression	4 + 9†
13	M34	12/97	CI				1/25		1/28	bone: 1/4	continuous progression	12 + 5†

Treatment centers: three patients (04, 08, and 12) were treated in Wuerzburg, two in Mainz (patients 10 and 13), and the others in Erlangen.

Pretreatment therapy: PCI, polychemoimmuno. Preceding excisions and radiotherapies are not listed.

Metastases at study entry: the number and diameter of the largest metastases present at study entry are listed (number/diameter in millimeters). m, multiple (>3 metastases).

Survival: (since onset of stage IV and as of 5 August 1999) is listed as months since onset stage IV until study entry + number of months since study entry. †Patient deceased.

*CNS metastases were regressing at study entry after gamma knife treatment.

*Developed (in part) after study entry.

*Determined by autopsy.

*Sudden death from bleeding into CNS metastasis on visit 8.

*The regressions of lung metastases in patients 06 and 07 were documented at a staging 3 mo after visit 8.

medlast., mediastinum; pancr., pancreas.

Table II. Study Design

Activities	Screen	Leuka pheresis	Vacc. #1 3 Mio s.c. 3 Mio i.d.	Vacc. #2 3 Mio s.c. 3 Mio i.d.	Vacc. #3 3 Mio s.c. 3 Mio i.d.	Vacc. #4 6 Mio i.v.	Vacc. #5 12 Mio i.v.	Final Evaluation
Clinical visit	1	2	3	4	5	6	7	
Day	-28/-14	-9	+1	+14	+28	+42	+56	+70
Vaccination			X	X	X	X	X	
Multitest Merieux DTH to Mage-3A1 peptide-loaded DC	X		X		X		X	X
Recall antigen proliferation		X						X
CTLp analysis		X				X		X
ELISPOT Mage-3A1		X	X	X	X	X	X	X
ELISPOT recall antigen		x	x	x	x	x	x	x

X, prespecified in the protocol as obligatory; x, optional.

neous vaccination sites that increased with the number of vaccinations. In 9/11 patients, strong DTH reactions (induration >10 mm in diameter) were noted to DCs carrying a recall antigen (Fig. 1). Elevation of body temperature (grade I and II fever) was observed in most (9/11) patients and was also related to pulsing DCs with recall antigen. The most striking example was patient 02, who progressively developed fever (up to 40°C) upon successive vaccinations but did not show a rise in body temperature when TT was omitted for the final (fifth) vaccination. We observed slight lymph node enlargement, clinically in 63% and by sonography in 83% of patients, after the intracutaneous DC injections. Interestingly, these were delayed, being inapparent during the 2 d of monitoring after vaccinations but detected when patients were investigated again the day before the next vaccination (Table II).

Immunological Responses

Boosting of Recall Antigen-specific Immunity. PBMCs that had been frozen before vaccination and 14 d after vaccination 5 were thawed and assayed together, as specified in the protocol (Table II). In most patients, a significant boost of antigen-specific immunity developed to TT (and tuberculin in patient 10) ($P < 0.004$; Fig. 2). Supernatants from the proliferative assays contained large amounts of IFN- γ (mean 1,679 pg/ml, range 846–4,325) but little IL-4 (IFN- γ /IL-4, 317:1). In five patients, we studied the kinetics of the immune response to TT by IFN- γ ELISPOT analysis. We found an increase after the intracutaneous vaccinations ($P < 0.02$) but a peculiar decrease after the intravenous vaccinations ($P < 0.008$; Fig. 3). Thus, comparing recall immunity before and after all five vaccinations (Fig. 2) as prespecified in the protocol (Table II) obviously underestimated the extent of boosting.

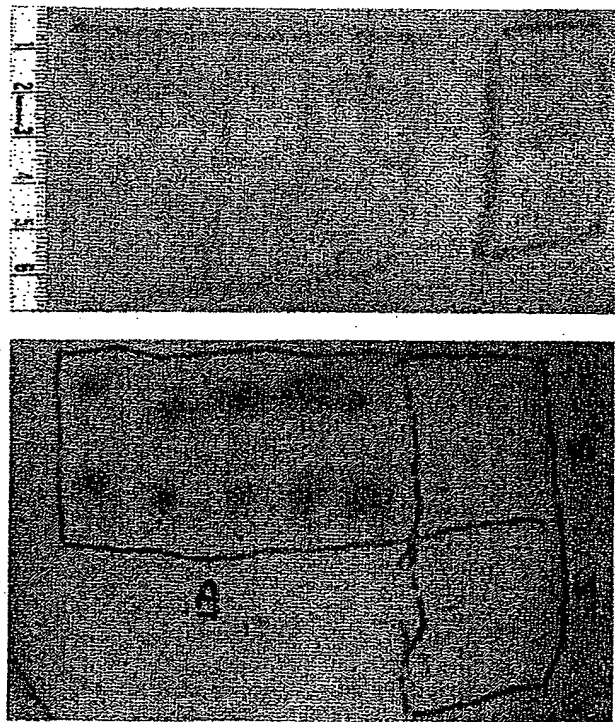


Figure 1. Local reactions to DCs carrying Mage-3A1 peptide and TT at the intradermal and subcutaneous vaccination sites in patient 09 (24 h after vaccination 2; top panel) and 02 (48 h after vaccination 3; bottom panel). Erythema at the 10 intradermal (left) and 2 subcutaneous (right) vaccination sites was followed by induration >10 mm in diameter (with secondary purpura in patient 02). These local reactions represent strong DTH reactions to DCs carrying TT, as such strong reactions did not occur in response to unpulsed DCs or DCs pulsed with Mage-3A1 peptide alone in DTH tests I–III (Table II; reactions not shown).

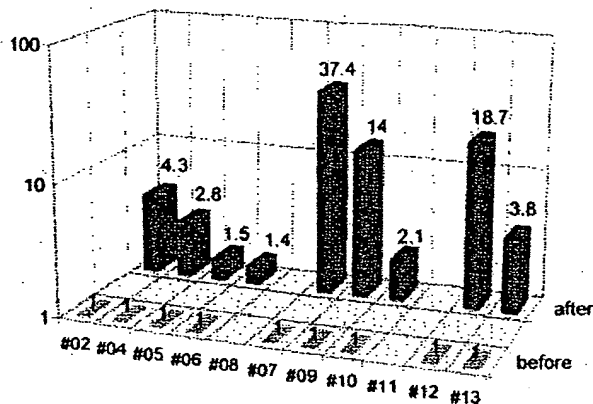


Figure 2. Recall antigen-specific immunity (tuberculin in patient 10; TT in all others) as assayed by antigen-specific proliferation. The cpm values determined after therapy (14 d after vaccination 5) are shown as multiples of pretherapy cpm values. Absolute cpm (cpm with recall antigen minus cpm without antigen) after therapy was 68,917 in patient 02, 85,225 in patient 04, 16,759 in patient 05, 7,913 in patient 06, 16,367 in patient 07, 107,923 in patient 09, 22,790 in patient 10, 4,507 in patient 12, and 1,831 in patient 13 (SEM for all measurements was <20%). Patients 08 and 11 could not be evaluated due to shortage of cells after therapy.

Expansion of Mage-3A1-specific CTLp. Aliquots of PBMCs, frozen before the first and after the third and fifth vaccinations, were thawed at the same time (Table II) and subjected to a semiquantitative recall assay for CTLp (reference 17; Fig. 4). Before vaccination, CTLp frequencies were low or undetectable. In 8/11 patients, we found a significant expansion of Mage-3A1-specific CTLp as indicated by the increase (mean, eightfold; $P < 0.008$) of positive microcultures in the multiple microculture procedure employed for the semiquantitative assessment of CTLp (17). Interestingly, in six patients, the CTLp frequencies were maximal after the three intracutaneous vaccinations ($P < 0.0013$) but then decreased after the two additional intravenous vaccinations in all but one of these patients ($P < 0.026$). Only in 1/11 patients did we observe an increase of CTLp to an irrelevant PB1 influenza peptide that served as a specificity control (not shown).

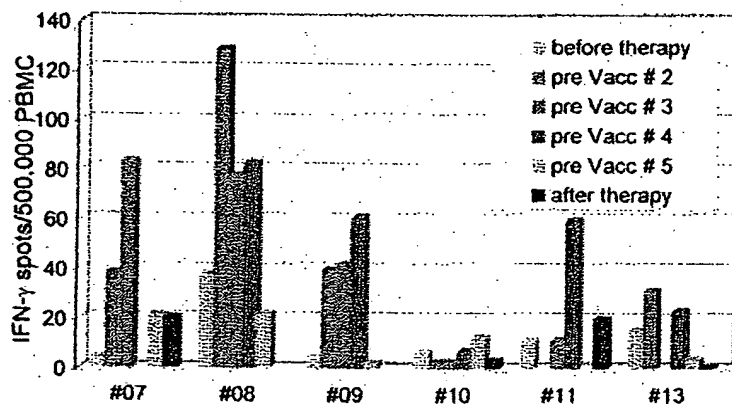


Figure 3. Kinetic analysis of immunity to recall antigens as assessed by TT-specific IFN- γ ELISPOT (SEM for all measurements was <20%). Blood was drawn (see Table II, Study Design) before the first DC vaccination and then every 14 d just before administration of the next DC vaccination (e.g., pre Vacc # 2 means immediately before vaccination 2, i.e., 14 d after vaccination 1), and finally after therapy. Time points at which vaccinations were not performed lack bars. Note the increase after the intracutaneous vaccinations and the decline upon the two vaccinations after intravenous ones. Patient 10, who received tuberculin-pulsed DCs, exhibited no significant change in the TT-specific IFN- γ ELISPOT as expected.

ELISPOT Analysis for IFN- γ -releasing, Mage-3A1-specific T Cells. We also tried to detect Mage-3A1-specific CTL effectors in uncultured fresh, nonfrozen PBMCs by performing ELISPOT analyses at 14-d intervals on all patients. A significant increase of Mage-3A1-reactive IFN- γ spot-forming cells was apparent only in patients 07 and 09 after the first and second vaccinations, respectively, but the frequency of Mage-3A1-specific effectors was very high ($\sim 5,000$ and $10,500/10^7$ CD8 $^+$ T cells; not shown).

DTH Test to Mage-3A1 Peptide-loaded DCs. Tests of DTH to Mage-3A1 peptide-loaded DCs yielded erythema and/or induration (>5 mm diameter) in 7/11 patients (not shown). The results were, however, equivocal due to the frequently observed background to nonpulsed DCs (up to 10 mm in diameter) and the variability from test site to test site.

Clinical Responses

At the end of the trial, i.e., ~ 2 wk after the fifth vaccination (Table II), we observed temporary growth cessation of some individual metastases, but more intriguingly, in 6/11 patients, complete regression of individual metastases in skin, lymph nodes, lung, and liver (Table I and Fig. 5). Resolution of skin metastases was found in three patients (Table I, patients 06, 07, and 08) and in two of them (06 and 07), it was preceded by local pain, itching, and slight erythema. The six regressing skin lesions of patients 06 and 07 (Table I) were also excised and examined by immunohistology. Clusters of CD8 $^+$ T cells were seen around and in the tumor, the latter often necrotic, suggesting an immune attack (Fig. 6).

In patients 06 and 08, the metastases excised at study entry (four and two, respectively) proved to be Mage-3 mRNA $^+$. However, all of the samples removed at the end (two and six, respectively) were Mage-3 mRNA $^-$, suggesting immunoselection for antigen-negative tumor cells. Remarkably, significant infiltration of CD8 $^+$ T cells was not found in any of these lesions.

Discussion

In deciding on the source of DCs for this phase I trial, we selected *mature*, monocyte-derived DCs for the follow-

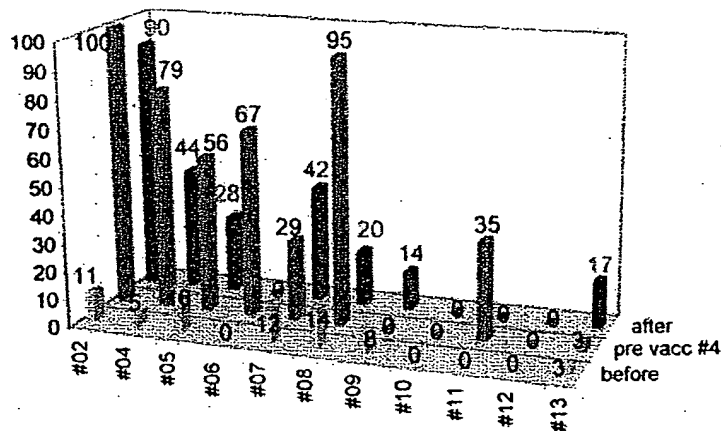


Figure 4. Mage-3A1 CTLp frequency analysis as assessed by semiquantitative recall assay. The y-axis and the numbers above the bars indicate the percentage of positive wells found before vaccination 1, before vaccination 4 (14 d after vaccination 3), and after therapy (usually 14 d after vaccination 5).

ing reasons. Monocyte-derived DCs currently represent the most homogenous and potent DC populations, with several defining criteria and quality controls (12, 13, 21). The method for generating production of these DCs is very reproducible and allows the cryopreservation of large numbers of cells at an identical stage of development (12, 13). Furthermore, these DCs can be produced in the absence of potentially hazardous FCS (12, 13, 21). FCS exposure also leads to large syngeneic T cell responses in culture, so their clinical use (11) might produce nonspecific immunostimulatory effects. Unlike other investigators (9–11), we chose to use mature rather than immature DCs for our first melanoma trial. The DCs that have been used with efficacy in animal experiments were primarily mature (3, 8). Mature DCs are much more potent in inducing CTL and Th1 responses in vitro (reference 22 and Jonuleit, H., A. Gieseke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk, manuscript in preparation), and the DCs are also resistant to the immunosuppressive effects of IL-10 (23) that can be produced by tumors (24–26). Mature DCs also display an extended half-life of antigen-presenting MHC class I (26a) and class II molecules (27). Finally, mature DCs have a high migratory activity (21) and express CCR7 (28), a receptor for chemokines produced constitutively in

lymphoid tissues (28). Mature DCs, as used in this cancer therapy trial, have recently also been shown to rapidly generate broad T cell immunity in healthy subjects (28).

Mature DCs were loaded with only one melanoma peptide, Mage-3A1, to avoid uncertainties regarding loading of DCs with multiple peptides (11) of varying affinity and off rate. Successful loading was verified with a Mage-3A1-specific CTL clone and ELISPOT analysis (not shown). The Mage-3A1 peptide (15) was selected for several reasons. It is essentially tumor specific (2) and expressed in tumors other than melanoma (2), and the Mage-3A1 epitope is likely a rejection antigen (14). Moreover, the Mage-3A1 CTLp frequency is exceedingly low in noncancer patients (reference 18; 0.4–3 per 10^7 CD8⁺ T cells) as well as in cancer patients, even after peptide vaccination (14). Thus, any induction or boost of Mage-3A1 CD8⁺ T cell responses would indicate a significant superiority in the adjuvant capacities of DCs.

DTH assays with peptide-pulsed DCs were carried out as described by Nestle et al. (11) to detect Mage-3A1 immunity (not shown). However, we did not detect unequivocal DTH. This was due to the frequently observed background to nonpulsed DCs (possibly due to cytokine production by DCs) and the noteworthy variability from test site to test site. As Mage-3A1-specific T cells are CD8⁺

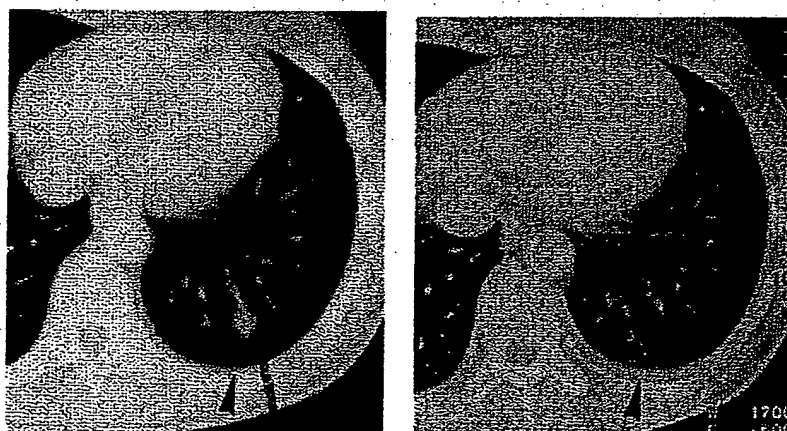


Figure 5. Regression (arrows) of a globular (13 mm in diameter) lung metastasis in patient 07 that was then no longer detectable in serial 6-mm-thick computed tomography scans.

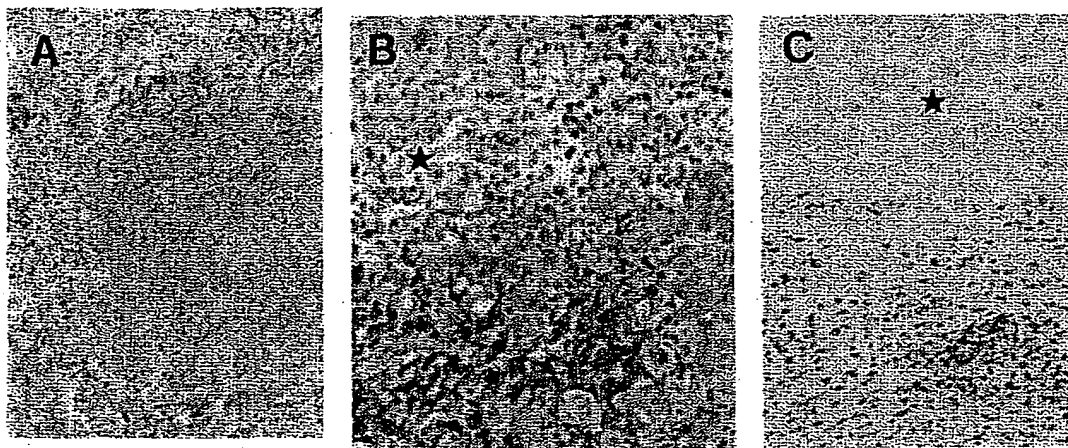


Figure 6. Regressing subcutaneous metastases in patient 06 display a CD8⁺ lymphocytic infiltrate (alkaline phosphatase/antialkaline phosphatase immunohistochemical staining with anti-CD8 mAb) that surrounds (A) and infiltrates (B) the tumor. Areas of damaged (B, ★) and necrotic (C, ★) melanoma cells are obvious in the vicinity of the CD8⁺ T cell infiltrate. The metastasis expressed Mage-3, as demonstrated by RT-PCR (data not shown). Magnifications: A, 100; B, 250; C, 160.

T cells and DTH assays typically detect primed CD4⁺ T cells, we suspect that DTH to MHC class I peptide-pulsed DCs may also for this reason prove not to be a sensitive or reliable way to monitor specific CD8⁺ T cell-mediated immunity.

In contrast, we found sizable expansions of Mage-3A1-specific CTL precursors in PBMCs from a majority (8/11) of patients ($P < 0.008$; Fig. 4). This is an important proof of the principle of DC-based immunization, and it is also significant from the point of view that tumors can induce tolerance or anergy. It is very promising that CTLp expansions can be induced in far advanced and heavily pretreated stage IV melanoma patients. However, active Mage-3A1-specific effectors were generally not observed in ELISPOT assays, except for in two patients with high frequencies ($>5,000/10^7$ CD8⁺ T cells). Perhaps active CD8⁺ effectors were rapidly sequestered in the numerous metastases, as suggested by the biopsy studies illustrated in Fig. 6. An alternative explanation is that looking for effectors in peripheral blood 14 d after a preceding vaccination might simply be too late.

Interestingly, in six patients, CTLp had increased to their highest levels after the three intracutaneous vaccinations ($P < 0.0013$) and then decreased ($P < 0.026$) with subsequent intravenous immunizations (Fig. 4). The decrease in CTLp might be due to emigration of activated Mage-3-reactive CTLs into tissues, tolerance induction, or clonal exhaustion via the intravenous route. We also observed decreased responses to recall antigens in the five patients that we studied before and after intravenous vaccination (Fig. 3). The effect of the intravenous route requires additional study, as it may be counterproductive. In contrast, our results clearly demonstrate that the intracutaneous route is effective, so that the less practical intranodal injection propagated by other investigators (11) does not seem essential. It will, however, be necessary to compare subcutaneous and intradermal routes to find out if one is superior.

We found regression of individual metastases in 6/11 patients when patients were staged 14 d after the fifth vaccination (Table I). This percentage of responses was unexpected in far advanced stage IV melanoma patients who were all progressive despite standard chemotherapy and even chemimmunotherapy. In the study by Nestle et al. (11), chemotherapy was only given to 4/16 melanoma patients, and objective tumor responses were observed in 5/16. Therefore, we attribute the regressions to DC-mediated induction of Mage-3A1-specific CTLs. This interpretation is supported by the heavy infiltration with CD8⁺ T cells of regressing but not nonregressing (skin) metastases. The observation that all of the metastases in patients 06 and 08 that were excised at the end of the study were Mage-3 mRNA⁺ (whereas those removed at the onset were uniformly positive) suggests immune escape of and selection for Mage-3 antigen-negative tumors. Immune escape might also have been responsible for the lack of tumor response in those nonresponders that had mounted a Mage-3A1-specific CTL response.

After the end of the trial, surviving patients received further vaccinations with DCs and several tumor peptides (Mage-1, tyrosinase, and Mage-3) that were no longer part of the protocol. It is encouraging that 5/11 patients are still alive (Table I) 9–17 mo after study entry, as the expected median survival in patients progressive after chemo(immuno)therapy is only 4 mo (29, 30). One of the initial responders (patient 06) has recently experienced a complete response and has now been disease free for 2 mo. It is interesting that Marchand et al. (14) have also observed that regressions, once they have started, proceed slowly and may take months to complete.

In conclusion, the use of a defined DC vaccine combined with detailed immunomonitoring provides proof that vaccination with mature DCs expands tumor-specific T cells in advanced melanoma patients. In addition, we have found some evidence for the direct interaction between

CD8⁺ CTLs and tumor cells as well as for escape of antigen-negative metastases. We are convinced that DC-mediated immunization can be intensified further to reveal the presence of expanded populations of effector cells. Efficacy might be increased at the level of the DC, e.g., by optimizing

variables such as DC maturational state, route, dose, and schedule or by improving the short life span of DCs in vivo (31, 32); at the level of the T cell, e.g., by providing melanoma-specific CD4⁺ T cell help (33, 34) or IL-2 (35); and by treating patients earlier in their disease course.

We are grateful to all patients for their confidence and cooperation. We appreciate the support of J. Knop, Head of the Department of Dermatology in Mainz. We thank our colleagues H.B.-R. Balda, H. Hintner, F.S.M. Meurer, and C.R. Neumann for referring patients and T.L. Diepgen for statistical analysis. We are particularly grateful to T. Boon and P. van der Bruggen, who helped us in many ways, and to A. Knuth and T. Woelfel for help in establishing the semiquantitative CTL and ELISPOT assays, respectively. We are obliged to the Protocol Review Committee and Office of Clinical Trials Management of the Ludwig Institute for Cancer Research, in particular H.F. Oettgen and E. Hoffman, for their suggestions on improving the protocol and for supervising the trial.

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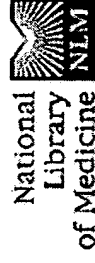
Address correspondence to Gerold Schuler, Dermatologische Klinik mit Poliklinik, Hartmannstr. 14, D-91052 Erlangen, Germany. Phone: 49-9131-85-0, ext. 33661; Fax: 49-9131-85-6175; E-mail: schuler@derma.med.uni-erlangen.de

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[JOURNAL SCIENCE](#)
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Immunomodulation by an inhibitor of S-adenosyl-L-homocysteine hydrolase: inhibition of in vitro and in vivo allogeneic responses.

Wolos JA, Frondorf KA, Babcock GF, Stripp SA, Bowlin TL.

Marion Merrell Dow Research Institute, Cincinnati, Ohio.

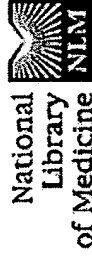
The response of murine T cells to MHC class II determinants on allogeneic cells induces helper T cell activation and the development of cytotoxic T cells. We have recently established that an S-adenosyl-L-homocysteine hydrolase inhibitor, (Z)-5'-fluoro-4',5'-didehydro-5'-deoxyadenosine (MDL 28,842), is a potent immunosuppressive agent which selectively inhibits T cell activation. In this report we characterize the effect of MDL 28,842 on in vitro and in vivo models of transplant rejection. In vitro, MDL 28,842 inhibited the generation of cytotoxic T cells in the murine mixed lymphocyte reaction with an IC50 of less than 0.1 microM. MDL 28,842 (1.0 microM) totally inhibited the generation of cytotoxic T cells when added up to 3 days after the initiation of culture with no apparent cell toxicity. In vivo, MDL 28,842 given by gavage at 5.0, 2.5, or 1.0 mg/kg/day inhibited the increase in popliteal lymph node weight induced by injection of allogeneic spleen cells into the footpad. MDL 28,842 was also evaluated in a model of graft rejection. Skin allografts on animals given MDL 28,842 at 5 mg/kg/day (ip) for the first 6 days following transplantation survived for 12.2 days, compared to 8.7 days for control animals. Cyclosporin A (CSA) given at 5.0 mg/kg/day did not prolong graft survival. The combination of MDL 28,842 and CSA was not any more effective than MDL 28,842 alone. Based on these findings, we suggest that MDL 28,842 is useful in the prevention of allograft rejection.

PMID: 8330316 [PubMed - indexed for MEDLINE]

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Tepoxalin, a novel immunomodulatory compound, synergizes with CsA in suppression of graft-versus-host reaction and allogeneic skin graft rejection.

Fung-Leung WP, Pope BL, Chourmouzis E, Panakos JA, Lau CY.

R. W. Johnson Pharmaceutical Research Institute, Don Mills, Ontario, Canada.

Tepoxalin, a dual 5-lipoxygenase and cyclooxygenase inhibitor with nonsteroidal antiinflammatory effects, has recently been shown to suppress NF kappa B transactivation and inhibit T cell proliferation via a mechanism very different from cyclosporine (CsA). In this report, we demonstrate that this novel immunosuppressive effect of tepoxalin is manifested in in vivo transplantation models. Tepoxalin suppressed murine spleen cell proliferation in a mixed lymphocyte reaction (MLR) with an IC50 of 1.3 microM. Coadministration of tepoxalin and CsA in MLR cultures showed an additive inhibitory effect. Oral administration of tepoxalin at 12 mg/kg/day to mice suppressed local graft-versus-host (GVH) responses by about 40% (n = 10). Combination of tepoxalin and CsA at suboptimal doses synergized their immunosuppressive effects on GVH responses (n = 20). In skin transplantation, the median survival time of allogeneic BALB/cByJ (H-2d) mouse skin grafted onto C3H/HeJ (H-2 kappa) mice was 10.5 days (n = 8), and was prolonged to 15.0 days (n = 9) for recipient mice administered tepoxalin at 50 mg/kg/day. Coadministration of suboptimal doses of tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) prolonged skin graft rejections dramatically (55% of the grafts survived for more than 40 days, n = 9). Taken together, these results demonstrate that tepoxalin is a potent immunomodulatory compound that, when combined with CsA, provides synergistic immunosuppressive activity. The fact that tepoxalin and CsA act on different transcription factors, NF kappa B and NFAT respectively, might explain the synergistic suppressive effects when both compounds were used. Tepoxalin could be an important addition to the cohort of immunosuppressive therapies currently used in solid organ and bone marrow transplantations.

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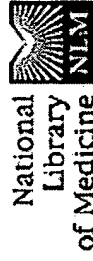
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RELNEVIEW SCIENCE
FULL-TEXT ARTICLE

Combination therapy with a CD4-CDR3 peptide analog and cyclosporin A to prevent graft-vs-host disease in a MHC-haploidentical bone marrow transplantation model.

Townsend RM, Gilbert MJ, Korngold R.

Kimmel Cancer Institute, Jefferson Medical College, Philadelphia, Pennsylvania 19107, USA.

Graft-versus-host disease (GVHD) is a major complication associated with allogeneic bone marrow transplantation (BMT). Cyclosporin A (CsA) has been used as the basis for most immunosuppressive regimens for the prevention of GVHD, but has exhibited only limited effects and is hampered by nephrotoxicity, neurotoxicity, and hepatotoxicity. Previously, we showed that rD-mPGPtide, a structure-base designed peptide analog of the CDR3-like region of domain 1 of the murine CD4 molecule, suppressed the development of GVHD in a MHC-haploidentical murine BMT model when administered early in the course of disease. This peptide analog also inhibited T cell proliferation in mixed lymphocyte reactions (MLR) in vitro. The current results demonstrate that CsA and rD-mPGPtide exhibit an additive inhibitory effect on MLR. Furthermore, the use of CsA and rD-mPGPtide together for prevention of GVHD nearly doubled the median survival time of the mice compared to either agent alone. In addition, the combination therapy reduced the requirement for habitual administration of CsA. Therefore, the use of a CD4-CDR3 peptide can complement and potentiate the immunosuppressive effects of CsA in the prevention of GVHD following allogeneic BMT.

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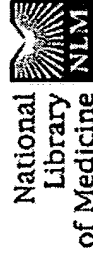
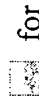
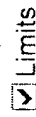
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Inhibitory effect of a CD4-CDR3 peptide analog on graft-versus-host disease across a major histocompatibility complex-haploidentical barrier.

Townsend RM, Briggs C, Marini JC, Murphy GF, Korngold R.

Kimmel Cancer Institute, Jefferson Medical College, Philadelphia, PA 19107, USA.

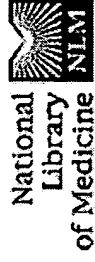
A structure-based designed peptide has been engineered to exhibit the same molecular surface as a portion of the CDR3-like region in domain 1 of the murine CD4 molecule. Earlier in vitro experiments indicated that this analog, known as rD-mPGPtide, inhibited T-cell proliferation in mixed lymphocyte reactions and blocked activation of both normal CD4+ T cells and T-cell lines after T-cell receptor triggering. In addition, rD-mPGPtide proved to be a potent inhibitor in vivo of CD4+ T-cell-mediated experimental allergic encephalomyelitis disease in the SJL mouse model. In this current report, we have evaluated the potential of rD-mPGPtide for suppressing the development of graft-versus-host disease (GVHD) in an irradiated major histocompatibility complex (MHC)-haploidentical murine bone marrow transplantation (BMT) model [(B6 x DBA/2)F1 -->(B6 x CBA)F1 (950 cGy)]. Our results indicated that early administration of rD-mPGPtide was effective in the inhibition of alloreactive responses of the donor T cells against the host and thus delayed or prevented the onset of GVHD. The median survival time of animals treated with rD-mPGPtide was enhanced as much as four-fold with as little as a single dose of peptide at the time of transplant. Decreased alloreactivity was indicated by phenotypic and functional analysis of positively selected thoracic duct lymphocytes 4 days after transplant and by histopathological examination of skin and gastrointestinal tissue samples 4 weeks later. Therefore, the administration of a CD4-CDR3 peptide is an efficacious approach against the development of GVHD during allogeneic BMT.

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FULL-TEXT ARTICLE

Immunomodulation by an adenylate cyclase activator, NKH477, in vivo and vitro.

Furukawa Y, Matsumori A, Hirozane T, Matsui S, Sato Y, Ono K, Sasayama S.

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Cyclic adenosine monophosphate (cAMP) is an intracellular second messenger which modulates T cell function. NKH477 is a direct adenylate cyclase activator derived from forskolin and now under clinical investigation as a positive inotropic agent. While the immunosuppressive effects of forskolin on lymphocytes have been reported, little is known about its effects in vivo. In this study, we investigated whether NKH477 has immunosuppressive effects in mice, namely on cardiac allograft survival, and on the generation of cytotoxic T lymphocytes (CTL), T cell proliferation in mixed lymphocyte reaction (MLR), and production of interleukin-2 (IL-2) in MLR and in mitogen response. We assessed the effects of standard immunosuppressant cyclosporin A (CsA) on IL-2 production and on allograft survival to estimate the intensity of rejection in this acute rejection model. Saline-treated C57BL/6 (H-2b) mice rejected DBA/2 (H-2d) cardiac allografts with a median graft survival time of 10 days. In contrast, median graft survival was prolonged to 12 and 15 days in mice treated with NKH477 at 1 and 3 mg/kg/day, respectively ($P < 0.01$ vs control). The equivalent dose of CsA (40 mg/kg/day) to the maintenance dose after clinical cardiac transplantation prolonged median graft survival time to 15.5 days, indicating that high dose of NKH477 was as efficacious as lower dose of CsA. Addition of NKH477 to the culture medium suppressed the generation of CTL, T cell proliferation in MLR, and production of IL-2 in MLR and in mitogen response. These results suggest that NKH477 exerts a beneficial effect on murine cardiac allograft survival by modulating T cell function.

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